UNIVERSITÀ DEGLI STUDI DI PALERMO

PhD school of Biomedicine and Neuroscience
Department of Biomedicine, Neurosciences and Advanced Diagnostics
SSD: BIO/09

BENEFICIAL EFFECTS OF PISTACHIO REGULAR INTAKE IN OBESITY-RELATED DYSFUNCTIONS

PhD STUDENT
DOTT. SIMONA TERZO

PhD COORDINATOR
PROF. FABIO BUCCHIERI

TUTOR
PROF. FLAVIA MULÈ

XXXIII CYCLE
YEAR OF DOCTORAL DEGREE ATTAINMENT 2019/2020

TABLE OF CONTENTS

Acknowledgments

Undertaking this PhD has been a truly life-changing experience for me and it would not have been possible to do without the support and guidance that I received from many people.

Firstly, I would like to thank my supervisor, Professor Flavia Mulè, for all theachings she has been giving me during these three years. Without her guidance and constant feedback this PhD would not have been achievable.

My deep appreciation goes to Professor Antonella Amato. Her excellent work has made an invaluable contribution towards my PhD. I am also grateful for her friendship, cordiality and continuous encouragments.

A very special thanks to other professor of Physiology Lab of STEBICEF Department: Professor Serio, Professor Zizzo, Professor Baldassano and Doctor Caldara for their precious advices, for feedback on my research, for their support to my work and making me feel at home.

I would also like to say a heartfelt thank you to my angel, Dad, who believed in it before me, and to my mum. Daniela and Salvo for their love and for encouraging me to follow my dreams.

And finally to Marco, who has been by my side throughout this PhD, living every single minute of it, and without whom, I would not have had the courage to embark on this journey in the first place.

Glossary

Below is a glossary of common abbreviations or acronysms that have been used throughout this thesis.

AD	Alzheimer's disease
AGEs	Glycation end products
APP	Amyloid precursor protein
AT	Adipose tissue
BACE1	Beta-site APP cleaving enzyme 1
BAT	Brown adipose tissue
BBB	Blood-brain barrier
BMI	Body-mass index
ChREBP	Carbohydrate response element binding protein
CRP	C-reactive protein
CVD	Cardiovascular diseases
DHA	Docosahexaenoic acid
DPPH	2, 2-diphenyl-1-picrylhydrazyl
EPA	Eicosapentaenoic acid
FA	Fatty acids
FAO	Food and agriculture organization
FAS	Fatty acid synthase
FAT-P	Fatty acid transport proteins
FDA	Food and Drug Administration
FFA	Free fatty acids
GLAP	Pentosidine and glyceraldehydes-derived pyridinium
GSK3	Glycogen synthase kinase-3
HDL	High-density lipoprotein
HFD	High fat diet
IDF	International Diabetes Federation
IKKβ	IκB kinase
IL	Interleukin
IR	Insulin resistance
IRS	Insulin receptor substrate
IκB-α	NFKB inhibitor α
JNK1	c-Jun N-terminal kinase 1
LDL	Low-density lipoprotein
LPS	Lipopolysaccharide
MDA	Malondialdehyde
MetS	Metabolic syndrome
MRI	Magnetic resonance imaging
mTOR	Mechanistic target of rapamycin
MUFA	Monounsaturated fatty acids
NAFL	Nonalcoholic fatty liver
NAFLD	Non-alcoholic fatty liver
NASH	Nonalcoholic steatohepatitis
NCEP: ATP III	National Cholesterol Education Program's Adult Treatment Panel III
NF-κB	Nuclear factor-κB
NO	Nitric oxide
PI3K	Phosphatidylinositol 3-kinase
PKC	Protein kinase C

PON1	Paraoxonase 1
PPAR-γ	Peroxisome proliferator-activated receptor γ
PUFA	Polyunsaturated fatty acids
RAGE	Receptor for advanced glycation endproducts
ROS	Reactive oxygen species
SAT	Subcutaneous adipose tissue
SCD1	Stearoyl-CoA desaturase
SOD	Superoxide dismutase
SREBP-1c	Sterol regulatory element-binding transcription factor-1c
STD	Standard diet
T2DM type 2	Diabetes mellitus
TGF-β	Transforming growth factor beta
TGs	Triglycerides
TLR4	Toll-like receptor 4
TNF-α	Tumor necrosis factor-α
VAT	Visceral adipose tissue
WAT	White adipose tissue
WHO	World Health Organization

PRELIMINARY CONSIDERATIONS

Obesity, one of the main health burden of the 21st century, is a metabolic disorder with a multifactorial origin, often associated with the development of a large number of health disorders, including diabetes, cardiovascular complications, cancer, hepatic dysfunction and brain impairments (Rusinek & Convit, 2014). Obesity is strongly linked to the metabolic syndrome, a cluster of diseases including hypertension, dyslipidemia and insulin resistance. Although there are genetic, behavioral, metabolic and hormonal influences, the obesity occurs when the calorie intake exceeds the energy expenditure, leading to fat accumulation. Classically, fat stands in subcutaneous and visceral adipose tissue, but also in other sites, such as lung, blood vessel wall, epicardium, kidney and bone marrow. Excessive fat storage in adipocytes initiates an inflammatory stress response characterized by the secretion of a large variety of chemokines responsible for a systemic chronic low-grade inflammation leading to the obesity-related disorders, such as insulin resistance (Nishida & Otsu, 2017). In fact, inflammation and oxidative stress are the links between the different symptoms of metabolic syndrome and anti-inflammatory and antioxidant interventions may attenuate the disorders characterizing the metabolic syndrome (Merone & McDermott, 2017; Gregório et al., 2016).

A broad range of strategies have been recommended to reduce the increasing prevalence of obesity, including regular physical activity, meal replacements, micronutrient supplementation and dietary intake of fruits and vegetables. Dietary consumption of foods rich in monounsaturated fatty acids (MUFA), ω-3 polyunsaturated fatty acids (PUFA), antioxidants, micronutrients, phytochemicals, and probiotics has been found to be helpful in maintaining body weight and reducing the incidence of metabolic diseases (Arora et al., 2013; Gonzalez-Castejon & Rodriguez-Casado, 2011).

Also the daily nut consumption has been reported to improve dysmetabolic conditions such as obesity, type 2 diabetes mellitus (T2DM), and related cardiovascular diseases (CVD) (Tan et al., 2014). Among nuts, Pistachio is the healthiest one because of its nutritional profile and bioactive compound content (Terzo et al., 2019). In fact, compared to other nuts, dry roasted pistachios have a lower fat content, mainly characterized by MUFA and PUFA. Pistachios also contain significant amounts of minerals and vitamins such as vitamin A, vitamin E (especially γ-tocopherol), vitamin C, vitamin B, vitamin K, and folate. Pistachios are the nuts with the highest content of phytosterols, including stigmasterol, campesterol, and β-sitosterol. Moreover, pistachios are rich of lutein, zeaxanthin (xanthophyll carotenoids) and phenolic compounds, including anthocyanins, flavonoids,

and proanthocyanidins. As consequence of composition, pistachios have a considerable antioxidant and anti-inflammatory capacity.

However, although regular pistachio intake could induce better benefits than other nuts, the potential beneficial properties of its regular consumption on obesity-related dysfunctions have not been explored sufficiently yet.

The present research was undertaken in the attempt to examine whether regular pistachio consumption can improve the obesity-related metabolic dysfunctions. In particular, we explored the impact of pistachio intake on different aspects of metabolic syndrome, such as glucose and lipid homeostasis, hepatic steatosis, adiposity, visceral and systemic inflammation, dysbiosis, neurodegeneration and brain oxidative stress. In this view, we used mice with diet-induced obesity. Indeed, C57BL/6J mouse, when allowed *ad libitum* access to high-fat diet (HFD), develops not only obesity, hyperglycaemia, insulin resistance, hepatic steatosis, systemic inflammation (Collins et al., 2004), but also neuroinflammation and neurodegeneration (Nuzzo et al., 2015).

The animals were divided in groups, which were fed differently for 16 weeks, as it follows: 1. Lean group, fed a standard diet (STD) 2. HFD group, fed a HFD (untreated HFD mice) 3. HFD-P group, fed a HFD supplemented with pistachio. HFD-P was custom prepared by Mucedola S.r.l (PF4215/C; R&S 34/16) by substituting 20% of HFD caloric intake with pistachio (180 g/Kg HFD). At the end of the 16th weeks, the animals were sacrificed and different organs and tissues were opportunely explanted. Different parameters were evaluated and compared among three animal groups.

The results are presented in relation to the original published papers; therefore, methods are described within each chapter.

Specifically, the first article focuses on pistachio consumption and hyperglycemia, dyslipidemia, hepatic steatosis and adiposity. Micro-computed tomography scans were performed to assess the volumes of the visceral adipose tissue and subcutaneous adipose tissue depots. RT-PCR was also used to analyze the expression of peroxisome proliferator-activated receptor γ (PPAR-γ), fatty acid transport proteins (FAT-P), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1), and sterol regulatory element-binding transcription factor-1c (SREBP-1c) in liver and adipose tissue.

The second article concerns the impact of regular pistachio intake on obesity-associated inflammation and gut microbiota composition in HFD mice. ELISA Kit was used to analyze

systemic inflammation markers and RT-PCR and immunohistochemistry analysis were used to reveal the adipose and hepatic tissue inflammation. In attempt to elucidate, an eventual contribution of the gut microbiota to the beneficial pistachio effects, fecal samples were used for DNA extraction and hypervariable V3-V4 regions of the 16S rRNA gene amplification. The resulting amplicons were sent to the BMR Genomics company s.r.l. (Padua-Italy) for the sequencing of the amplified 16S ribosomal gene region, by Next Generation Sequencer (NGS) technique. Ussing chamber assay was used to evaluate intestinal permeability.

The third article investigated the neuroprotective effects of pistachio intake in HFD mice. Metabolic parameters (oxidative stress, apoptosis, and mitochondrial dysfunction) were analyzed in mouse brains by using specific assays and biomarkers.

Within the Introduction, it is my intention to provide an overview about the different disorders characterizing the metabolic syndrome and to provide a state of art about pistachio health benefits.

PUBLICATIONS

The following publications have arisen due to work performed during my PhD candidature and are the basis of this thesis:

Journal Articles

- **Terzo S**, Caldara GF, Ferrantelli V, Puleio R, Cassata G, Mulè F, Amato A. Pistachio consumption prevents and improves lipid dysmetabolism by reducing the lipid metabolizing gene expression in diet-induced obese mice. *Nutrients*. 2018; 10:1857.
- **Terzo S**, Baldassano S, Caldara GF, Ferrantelli V, Lo Dico G, Mulè F, Amato A. Health benefits of pistachios consumption. *Nat Prod Res.* 2019; 33:715-726.
- **Terzo S**, Mulè F, Caldara GF, Baldassano S, Puleio R, Vitale R, Cassata G, Ferrantelli V, Amato A. Pistachio consumption alleviates inflammation and improves gut microbiota composition in High Fat Diet fed mice. *Int. J. Mol. Sci.* 2020; 21: E365.
- Nuzzo D, Galizzi G, Amato A, **Terzo S**, Picone P, Cristaldi L, Mulè F, Di Carlo M. Regular Intake of Pistachio Mitigates the Deleterious Effects of a High Fat-Diet in the Brain of Obese Mice. *Antioxidants (Basel).* 2020; 9(4):317.

Abstracts

- S. Terzo, F. Mulè, A. Amato. The impact of Sicilian pistachio on the obesity-related metabolic dysfunctions and microbiota composition in High-Fat-Diet mice. 12th Meeting of Young Researchers in Physiology. Anacapri (NA) – May 03-05 2018.
- S. Terzo, F. Mulè, Caldara G, Baldassano S, Vitale M, Puleio R, Ferrantelli V, Amato A. Pistachio prevents obesity-dysmetabolism in mice by modulating lipid metabolism genes and microbiota. 5° meeting 'Biotecnologie ricerca di base interdisciplinare traslazionale in ambito biomedico'. Palermo – July 05-06 2018.
- S Terzo, F. Mulè, A. Amato. Pistachio consumption reduces metabolic abnormalities in high-fat diet mouse model by modulating adipogenic gene expression. 69th SIF National Congress Italian Physiological Society. Florence – 19-21 September 2018.

1. Metabolic syndrome

Metabolic syndrome (MetS) is a complex, multi-factorial condition that predisposes an individual to some severe complications such as cancer, cardiovascular diseases, chronic kidney diseases and neurodegenerative disorders (Park et al., 2003). Several risk factors contribute to the constellation of abnormalities leading to MetS. These include elevated blood glucose, triglycerides, cholesterol levels, obesity, oxidative stress and increase in the blood pressure (Oda, 2012; Bonomini et al., 2015; Sasya M, et al., 2019).

MetS has emerged as a major health concern worldwide in the recent decades and likely, it is connected with the life-style changes in the modern era. Modern technological advancements have produced dramatic changes in the way of life of individuals, including calorie intake and energy consumption (Robbins et al., 2014).

***Definitions*.** The definition of MetS has been constantly evolved over the years.

In the 1920s, MetS was first described by Kylin, a Swedish physician, as the clustering of hypertension, hyperglycaemia and gout (Kylin, 1923). Later, in 1947, Vague drew attention to upper body adiposity (android or male-type obesity) as the obesity phenotype that was commonly associated with metabolic abnormalities (type 2 diabetes and cardiovascular disease) (Vague, 1947). In 1998, in the attempt to achieve some agreement on definition and to provide a tool for clinicians and researchers, the World Health Organization (WHO) pr

oposed a set of criteria to identify metabolic syndrome (Alberti & Zimmet, 1998) (Table 1).

Table 1. Criteria proposed by World Health Organization to define metabolic syndrome

		Risk factors				
Agency	**Body weight**	**Insuline resistance**	**Lipids**	**Blood pressure**	**Glucose**	**Others**
World Health Organization (WHO), 1998	Waist/hip >0.9 (men) >0.85 (women) or body mass index (BMI) >30 kg/m2	Impaired glucose tolerance/Impaired fasting glycaemia/type 2 diabetes or lower insulin sensitivity + any 2 of the other factors	Triglycerides≥150 mg/dL and/or HDL <35 mg/dL (men) <39 (women)	≥140/90 mm Hg	Impaired glucose tolerance/Impaired fasting glycaemia/type 2 diabetes	Micro-albuminuria Urinary excretion rate >20 mg/min or albumin/creatinine >30 mg/g

Subsequently, the European Group for the Study of Insulin Resistance (Balkau & Charles, 1999) and the National Cholesterol Education Program's Adult Treatment Panel III (NCEP: ATP III) (Adult Treatment Panel III, 2001) formulated other criteria (Table 2 and 3).

Table 2. Criteria proposed by European Group for the Study of Insulin Resistance to define metabolic syndrome

		Risk factors				
Agency	**Body weight**	**Insuline resistance**	**Lipids**	**Blood pressure**	**Glucose**	**Others**
European Group for the Study of Insulin Resistance, 1999	Waist circumference ≥94 cm (men) ≥80 cm (women)	Plasma insulin >75th percentile	Triglyceride ≥ 150 mg/dL and/or HDL <39 mg/dL	≥140/90 mm Hg	Impaired glucose tolerance/fasting plasma glucose >110 mg/dL	None

Table 3. Criteria proposed by National Cholesterol Education Program's Adult Treatment Panel III to define metabolic syndrome

		Risk factors				
Agency	**Body weight**	**Insuline resistance**	**Lipids**	**Blood pressure**	**Glucose**	**Others**
National Cholesterol Education Program's Adult Treatment Panel III (NCEP: ATP III), 2001	Waist circumference ≥102 cm (men) ≥8 cm (women)	Any three of the five factors listed	Triglyceride ≥150 mg/dL and/or HDL <40 mg/dL (men) <50 (women)	≥130/85 mm Hg	>110 mg/dL	None

These definitions agree on the essential components (glucose intolerance, obesity, hypertension, and dyslipidaemia) but differ in the details and criteria. WHO definition was better suited as a research tool whereas the NCEP: ATP III definition was more useful for clinical practice. The NCEP: ATP-III definition is simpler for clinical practice. It requires only a fasting assessment of blood glucose, whereas the WHO definition requires an oral glucose tolerance test. Subsequently, the American Association of Endocrinology suggested that four factors should be the “identifying abnormalities” of the syndrome: elevated triglycerides, reduced HDL cholesterol, elevated blood pressure, and elevated fasting and post-load glucose (Table 4). Obesity was not included in the definition and given the mounting evidence that central obesity is a major risk factor for type 2 diabetes and cardiovascular disease this omission was rather surprising.

Because different definitions inevitably led to substantial confusion and absence of comparability between studies, the International Diabetes Federation (IDF) attempted to establish a unified definition for the metabolic syndrome. The major issue for the IDF definition was the fact that criteria used for obesity in different populations could be different. However, overweight and obesity

Table 4. Criteria proposed by American Association of Endocrinology to define metabolic syndrome

		Risk factors				
Agency	**Body weight**	**Insuline resistance**	**Lipids**	**Blood pressure**	**Glucose**	**Others**
American Association of Endocrinology, 2003	BMI ≥25 kg/m^2	Impaired glucose tolerance/Impaired fasting glycaemia + any of the other factors	Triglyceride ≥ 150 mg/dL and/or HDL <35 mg/dL (men) <39 (women)	≥130/85 mm Hg	Fasting plasma glucose 110–126 mg/dL; post-prandial 140–200 mg/dL	None

were redefined as body-mass index (BMI) and waist circumference, establishing different ranges between Asians and Europeans people (Table 5) (WHO/IASO/IOTF, 2000). Anyway, the International Diabetes Federation adopted waist circumference, defined with ethnicity specific values, as a *sine qua non* condition for the Metabolic Syndrome diagnosis.

Table 5. Criteria proposed by International Diabetes Federation to define metabolic syndrome

		Risk factors				
Agency	**Body weight**	**Insuline resistance**	**Lipids**	**Blood pressure**	**Glucose**	**Others**
International Diabetes Federation (IDF), 2005	Ethnicity based values for waist circumference >94 cm (Euro men) >80 cm (Euro women) >90 cm (Asian men) >80 cm (Asian women)	Not listed	Triglyceride ≥ 150 mg/dL and/or HDL <40 mg/dL (men) <50 (women)	≥130/85 mm Hg	>100 mg/dL	None

Therefore, according to the last definition of the International Diabetes Federation, metabolic syndrome include: central obesity (ethnicity specific waist circumference) plus any two parameters: *Raised triglycerides*>150 mg/dL (1·7 mmol/L)

Reduced HDL-cholesterol <40 mg/dL (1·03 mmol/L) in men <50 mg/dL (1·29 mmol/L) in women

Raised blood pressureSystolic ⩾130 mm Hg Diastolic ⩾85 mm Hg

Raised fasting plasma glucose ⩾100 mg/dL (5·6 mmol/L).

If above 5·6 mmol/L or 100 mg/dL, oral glucose tolerance test is strongly recommended, but is not necessary to define presence of syndrome (Alberti et al., 2005).

The new IDF criteria are not the final word, but hopefully will help identify people at increased risk, and through further research will lead to more accurate predictive indices.

1.1 Disorders underlying the metabolic syndrome and associated diseases

Obesity and increased waist circumference. Obesity is a heterogeneous condition deriving from genetic and lifestyle interactions (Albuquerque et al., 2015; Hopkins & Blundell, 2016). Epidemiological studies have documented that increased intake of energy and reduced consumption of high-fibre foods, as well as sedentary lifestyle, are among the major driving forces for the epidemic of obesity. Genome-wide association studies have identified several genes convincingly related to obesity risk, such as peroxisome-proliferator activated receptor-λ, lamin A/C, 1-acylglycerol-3-phosphate, O-acyltransferase, seipin, the β-2 adrenergic receptor, adiponectin and the melanocortin-4 receptor genes (Qi & Cho, 2008).

In the clinical practice, obesity is currently diagnosed by the assessment of BMI, defined as subject weight in kilograms divided by the square of his height in meters (kg/m^2). BMI is commonly used to classify overweight and obesity in adults. A BMI between 25 and 29.9 kg/m^2 defines overweight patients; BMI > 30 kg/m^2 defines obese patients. Among them, a BMI between 30 and 34.9 kg/m^2 identifies a class I obesity, between 35 and 39.9 kg/m^2 class II, and > 40 kg/m^2 a class III, also known as morbid obesity.

Obese subjects seem to have heterogeneous phenotypes, each one associated whit different degree of cardiovascular risk. Indeed, in human body two principal types of adipose tissue (AT) can be recognized: the brown adipose tissue (BAT), localized in supraclavicular and paravertebral regions, and the white adipose tissue (WAT). The latter includes subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT) surrounding abdominal organs. A phenomenon that has received increasing attention is the fact that body shape, and more specifically the regional distribution of adipose tissue, is at least as important, if not more important, than the total amount of body fat in predicting disease-causing complications that have been traditionally associated with obesity. In fact, VAT has been reported to be strictly associated with cardiometabolic risk, while SAT shows some protective metabolic features (Abraham et al., 2015). There are several possible explanations for these correlations. The visceral adipose tissue should produce a high flux of free fatty acids to the liver through the splanchnic circulation, while the subcutaneous adipose tissue would release lipolysis products into the systemic circulation without affecting hepatic metabolism (ie, glucose production, lipid synthesis, and secretion of prothrombotic proteins such as fibrinogen and plasminogen activator inhibitor 1) (Aubert et al., 2003).

Despite these potential differences in mechanisms related to excessive abdominal adipose tissue, the clinical diagnosis of the metabolic syndrome does not distinguish between increases in subcutaneous and visceral adipose tissues. Anyway, obesity is the main risk factor for development of insulin resistance and diabetes mellitus (Boles et al., 2017).

Glucose intolerance and insulin resistance. The most accepted and unifying disorder to describe the pathophysiology of the metabolic syndrome is insulin resistance. Insulin resistance is an impairment in which cells fail to respond normally to the hormone insulin. In this condition, pancreatic β cells increase the production of insulin, leading to high blood insulin (hyperinsulinemia) to compensate for the high blood glucose. During this compensated phase on insulin resistance, insulin levels are higher, and blood glucose levels are still maintained. If compensatory insulin secretion fails, then either fasting (impaired fasting glucose) or postprandial (impaired glucose tolerance) glucose concentrations increase. Eventually, type 2 diabetes occurs when glucose levels become higher as the resistance increases and compensatory insulin secretion fails. The inability of the β-cells to produce sufficient insulin in a condition of hyperglycemia is what characterizes the transition from insulin resistance to type 2 diabetes mellitus.

The insulin biological effects are subsequent to binding with specific membrane receptors, leading to autophosphorylation that enhances the receptor intrinsic tyrosine kinase activity. The next step in insulin signalling involves tyrosine phosphorylation of intracellular substrates including the insulin receptor substrate (IRS) family IRS-1, -2, -3, and -4. Tyrosine phosphorylated motifs on these IRS isoforms serve as binding sites for the SH2 domains contained in adaptor proteins such as the p85 regulatory subunit of phosphatidylinositol 3-kinase (PI3K). Downstream from PI3K, a series of serine kinases such as phosphoinositide-dependent kinase-1, Akt, and protein kinase C (PKC)-ζ are activated. This propagates insulin signalling to downstream effectors leading to biological actions of insulin including increased glucose transport, glycogen synthesis, and protein synthesis (Taniguchi et al., 2006).

In an insulin-resistance condition, there is overabundance of circulating fatty acids, derived mainly from adipose tissue, through the action of the cyclic AMP-dependent enzyme hormone sensitive lipase. Fatty acids also derive from the lipolysis of triglyceride-rich lipoproteins in various tissues by the action of lipoprotein lipase (Eckel, 1989). Since insulin has anti-lipolysis actions and it is the primary hormonal signal for energy storage into adipocytes (Jensen et al.1989), when insulin resistance develops, the increased lipolysis of stored triacylglycerol molecules produces more fatty acids, which could further inhibit the antilipolytic effect of insulin, creating additional lipolysis.

Upon reaching insulin sensitive tissues, excessive fatty acids create insulin resistance by modifying downstream signalling due to increased substrate availability.

In muscle, fatty acids can impair activation of PKC, a downstream effector of PI3K, compromising the ability to transmit the received insulin signal (Kim et al., 2002). Moreover, increased availability and oxidation of free fatty acids would increase intracellular levels of acyl-

CoA. The excessive generation of acyl-CoA or acyl-CoA- derivatives, such as ceramide, can diminish Akt1 activation, consequently the phosphorylation of a wide range of substrates, which act on the regulation of protein synthesis, including mechanis tic target of rapamycin (mTOR) and glycogen synthase kinase-3 (GSK3), results inhibited (Chavez et al., 2003]. In addition, studies on obese subjects (Kelley DE et al., 2002), patients with type 2 diabetes (Petersen et al., 2004) and the elderly people (Petersen et al., 2003) have identified a defect in mitochondrial oxidative phosphorylation that relates to the accumulation of triglycerides and lipid molecules in muscle.

In the liver, free fatty acids (FFA) and their intermediate metabolites accumulate in cell and activate PKC-ϵ. One possible target of PKC-ϵ is the c-Jun N-terminal kinase 1 (JNK1), a member of the mitogen-activated protein kinases. JNK1 has been shown to play a key role in the pathogenesis of fat-induced insulin resistance, possibly causing the serine phosphorylation of IRS-1 (Fig. 1). Serine-IRS1 phosphorylation represents a stop signal. The serine-phosphorylated IRS-1 complex blocks and detaches from the insulin receptor interrupting the interaction between IRS-1 and insulin receptor. Therefore, there is a block in the insulin-signalling pathway, which limits the ability of insulin to activate glycogen synthase (Samuel et al., 2004).

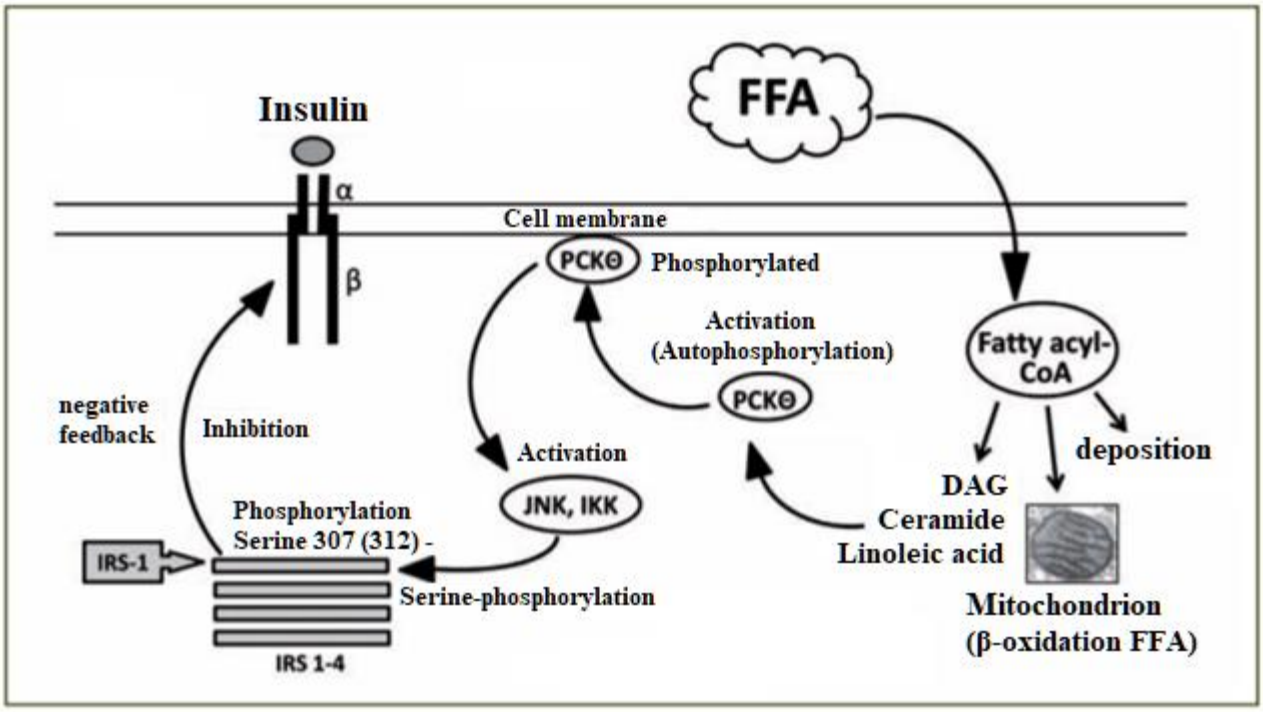

Fig. 1 Molecular mechanism of fat-induced insulin resistance in liver

Inflammation. Chronic positive energy balance leads to AT remodeling, characterized by an increase in size (hypertrophy) and in number (hyperplasia) of adipocytes associated with changes in adipose tissue immune cells (Makki et al., 2013). Both under physiological and

pathophysiological conditions, adipocytes can secrete *plus* than 50 cytokines, hormones and peptides, known globally as adipocytokines. They play an important role in regulation of energy homeostasis and in local and systemic inflammation (Liberale et al., 2017; Carbone et al., 2015). The hypertrophic adipocytes of obese individuals show an unbalanced adipocytokine production, with an increased secretion of pro-inflammatory mediators, such as leptin, resistin, interleukin (IL)-6, and tumor necrosis factor-α (TNF-α) (Fig. 2) (Liberale et al., 2017).

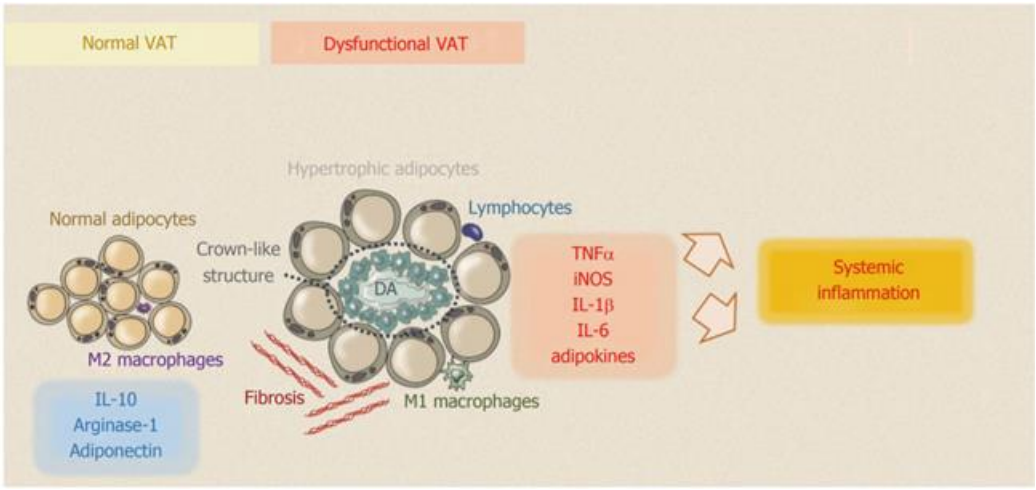

Fig. 2 The roles of AT immune cells in the development of obesity-induced inflammation

In particular, elevated serum TNF-α levels have been associated with obesity and insulin resistance, both of which major components of MetS (Tsigos et al., 1999). TNF-α production by macrophages within the adipose tissue increases in correspondence of adipose tissue mass. TNF-α causes phosphorylation and inactivation of insulin receptors in the adipose tissue, induction of lipolysis with production of FFA and inhibition of adiponectin release (Hotamisligil et al., 1994). IL-6, a cytokine produced by adipocytes and immune cells, has complex regulatory mechanisms (Fried et al., 1998). It acts on the liver, bone marrow, and endothelium, leading to increased production of acute phase reactants in the liver, including C-reactive protein (CRP). Several studies have demonstrated a correlation between high CRP levels and the development of MetS, diabetes, and CVD (Bastard et al., 2000). IL-6 also increases fibrinogen levels resulting in a prothrombotic state, and it promotes adhesion molecule expression by endothelial cells and activation of local renin-angiotensin system RAS pathways (Wisse, 2004). Therefore, there is a firm base of evidence linking the spectrum of dysfunctions charactherizing the metabolic syndrome to inflammation.

Cardiovascular alterations. Each component of the MetS is an independent risk factor for the development of CVD including microvascular dysfunction, coronary atherosclerosis,

hypertension, cardiac dysfunction and heart failure. Specifically, increased low-density lipoprotein (LDL) cholesterol levels, hyperglycemia, oxidative stress and inflammation, can cause vascular endothelial dysfunction resulting in atherosclerosis (Sena et al., 2013). The process is considered as follows (Fig. 3): (1) Vascular endothelial cells injured by oxidative stress or other factors express adhesion molecules and release cytokines and chemokines. (2) The chemokines attract monocytes from blood circulation to the injured area, and monocytes attach to the endothelium through interaction with adhesion molecules. (3) Monocytes penetrate the subendothelial space, differentiate, and mature into macrophages that release cytokines. When LDL cholesterol levels are high, LDL cholesterol infiltrates the subendothelial space and it is retained in the intima where it is oxidized or otherwise modified. (4) Macrophages take up and accumulate oxidized LDL cholesterol, leading to foam cell formation and atherogenesis. (5) Oxidized lipids trigger the secretion of various growth factors by the endothelium. Vascular smooth muscle cells of the media transform and migrate into the intima where they proliferate and actively produce extracellular matrix. These transformed vascular smooth muscle cells also take up oxidized LDL cholesterol and transform to form cells that contribute to atherogenesis. (6) On the other hand, the proliferation of vascular smooth muscle cells and an increase in extracellular matrix may cause intimal thickening and sclerosis.

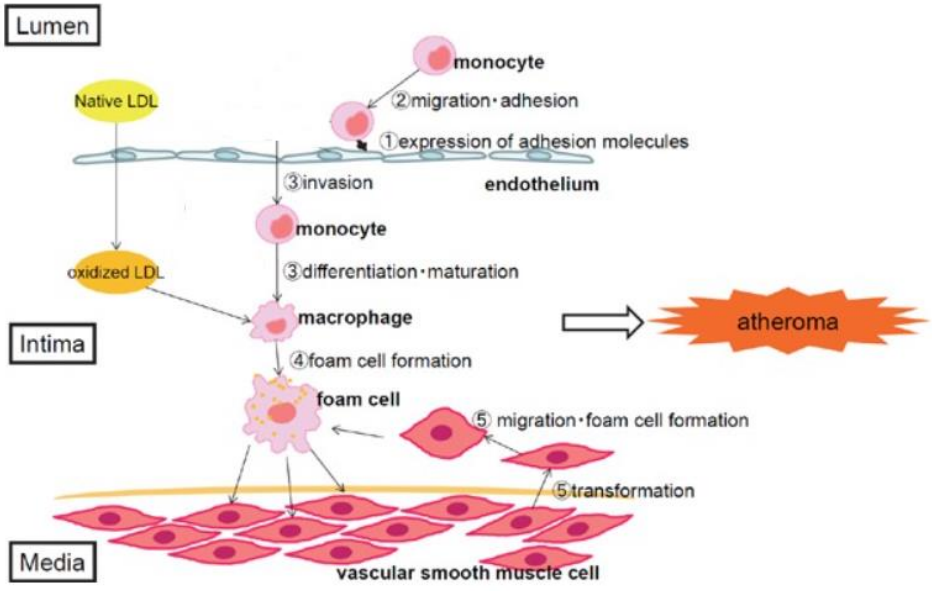

Fig. 3 Process of the formation of atherosclerotic lesions.

Endothelial dysfunction and alter hemodynamics throughout the body, can promote stiffness of the arteries and as consequence the elevation in blood pressure commonly observed in MetS (Lopes-Vicente et al., 2017). In addition to endothelial dysfunction in obesity condition, multiple potential mechanisms contribute to the development of higher blood pressure in including hyperinsulinemia, activation of the renin–angiotensin–aldosterone system, sympathetic nervous system stimulation, and abnormal levels of certain adipokines such as leptin (Hall et al., 2015). Hypertension on the other hand is a major risk factor for stroke, myocardial infarction, heart failure and chronic kidney disease.

Dysbiosis. The gut microbiota is currently one of the topics of highest interest in biomedical research, due to its potential key role in the development of many diseases. Although it is still being debated what constitutes a "healthy" gut microbiota composition, it has been widely established that dysbiosis, which refers to a disturbance in the amount and/or composition of "normal" gut microbiota, is strongly associated to a large number of common diseases. People presenting certain conditions, such as obesity and metabolic syndrome, consistently have lower bacterial diversity and altered composition compared to their healthy counterparts. In particular, in obese patients and in type 2 diabetes an increase in the number of Firmicutes to the detriment of Bacteroidetes has been reported (Castaner et al., 2018). Dietary interventions, such as high-fiber food, specific nutrients or prebiotics have been shown to promote bacterial diversity in the gut, and thus to induce an overall beneficial effect in the host.

Several evidence have linked the inflammatory state in metabolic syndrome to impaired gut barrier function and leakage of bacteria and/or bacterial components into the system, what is known as metabolic endotoxaemia (Piya, 2013). Indeed, in the gut, certain factors, such as a high fat diet, can promote the increase of Gram-negative bacteria that have lipopolysaccharide (LPS) on their outer membrane. Impairments of any intestinal barrier component (epithelial lining conjoined by junction proteins, thick mucus layers, luminal immunoactive components such as IgA, cytokines and mast cell proteases and gut-associated lymphoid tissue trained to discriminate commensals from pathogens) may lead to bacterial translocation and thus leakage and plasma increment of LPS. LPS, in turn, can activate inflammatory pathways by binding the CD14/Toll-like receptor-4 complex and consequently contribute to the insulin resistance and type 2 diabetes.

Non-alcoholic fatty liver disease. The hepatic manifestation of metabolic syndrome is non-alcoholic fatty liver disease (NAFLD). NAFLD includes a wide spectrum of liver damage, extending from hepatic fat deposition in the absence of inflammation to liver fibrosis and

hepatocarcinoma. Generally, two pathologically distinct conditions are identified: nonalcoholic fatty liver (NAFL) and nonalcoholic steatohepatitis (NASH). NAFL is characterized by simple liver steatosis, while NASH is characterized by the presence of steatosis and lobular inflammation with hepatocyte ballooning degeneration, with or without any fibrosis (Charlton et al., 2017). It is not clear yet whether NASH stems from NAFL or whether it represents a distinct entity characterized by a different prognosis.

The pathogenesis of NAFLD is multifactorial. Genetic factors cooperate with metabolic and environmental factors to promote the accumulation of fat in hepatocytes. In the last decade of the 20th century, the most corroborated theory was the "two hit pathogenesis". It stated that insulin resistance leads to triglycerides (TGs) deposition in the liver, thus steatosis, rendering it more susceptible to the action of second hits, such as oxidative stress, ATP depletion and endotoxins, and thus leading to inflammation, fibrosis and cancer. Nowadays, this theory has been replaced by the "multiple hit pathogenesis". This states that multiple etiopathogenic factors act in a parallel or in a sequential and somehow synergic way on a genetically predisposed subject, to cause NAFLD and thus defining the spectrum of the disease phenotype (Fig. 4) (Lonardo A et al., 2017). In other words, some patients will develop NAFL and consequently NASH, but others directly will present inflammation and fibrosis, depending on genetic and epigenetic factors.

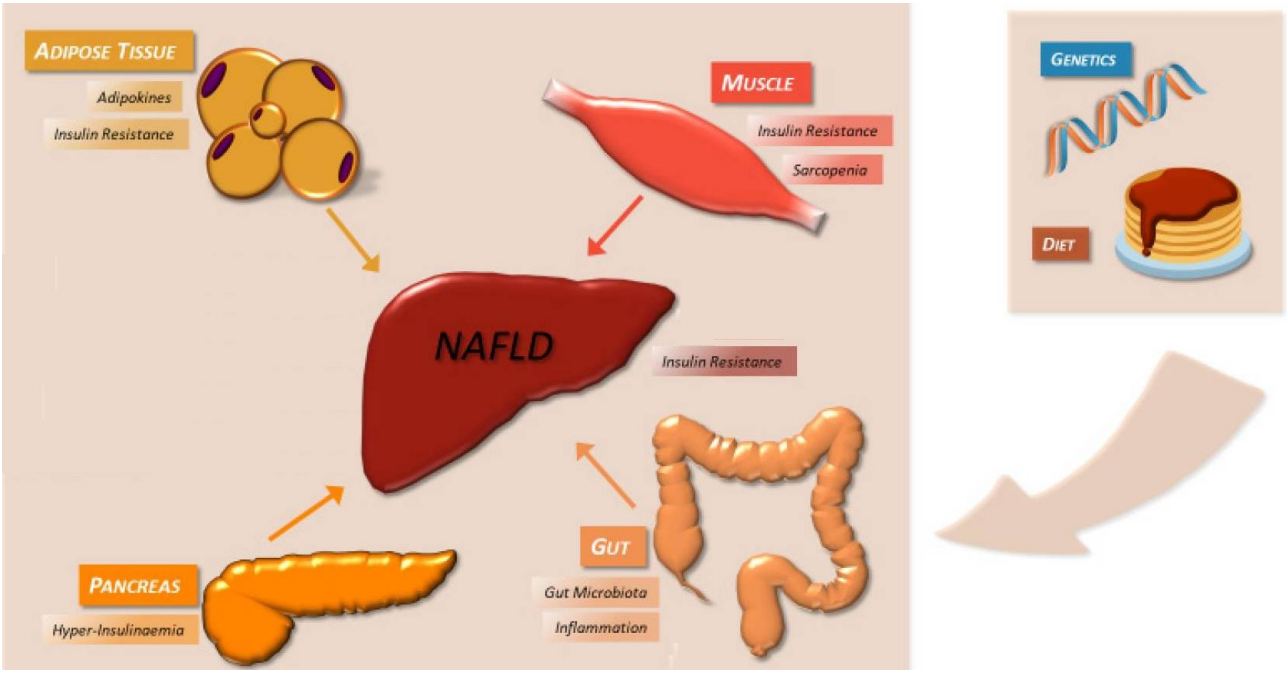

Fig. 4 Multiple-hit pathogenesis of NAFLD

The hallmark feature determining NAFLD is TGs accumulation in the liver, as a result of imbalance between fatty acid influx and efflux (Lonardo et al., 2017). An interesting study using

stable isotope labelling techniques demonstrated that 74% of hepatic TGs in NAFLD derives from exogenous sources, particularly from serum non-esterified fatty acids and from diet, while only 26% comes from hepatic *de novo* lipogenesis (McCullough et al., 2018). TGs excretion through very-low density lipoproteins increases in NAFLD, but it is not sufficient to compensate for the excessive inflow in the liver (Arab et al., 2018). NASH develops when physiological adaptive mechanisms of the liver are overwhelmed by the excessive influx of TGs, leading to lipotoxicity, inflammation, radical oxygen species (ROS) formation and hepatocellular dysfunction (McCullough et al., 2018). When an excessive amount of fatty acid reaches the hepatocyte, the mitochondria increase their utilization through beta-oxidation and oxidative phosphorylation. Furthermore, peroxisomes and endoplasmic reticulum also contribute to fatty acid oxidation. The drawback of this process is ROS formation that, in excessive quantity, consumes the antioxidant mechanisms of the cell and it leads to protein and lipid peroxidation, DNA damage and inflammation. Interestingly, mitochondrial function decreases in the advanced stages of NASH, generating a vicious cycle (Léveillé & Estall, 2019).

Insulin resistance has been traditionally identified as another key pathophysiological factor in NAFLD (Wainwright & Byrne, 2016). If insulin resistance develops, hormone-sensitive lipase is not suppressed and consequently the adipose tissue releases a great amount of non-esterified fatty acid into the bloodstream, leading to ectopic deposition of fat in organs such as the liver and pancreas. Characteristically, in NAFLD, steatosis is more abundant in the pericentral zone and it is rare in the periportal zone (Benedict & Zhang, 2017). Hijmans and collaborators proposed that insulin resistance leads to increased lypolisis in adipose tissue and thus an increased amount of non-esterified fatty acid arriving at the liver, specifically to periportal hepatocytes. These cells are consequently more prone to developing insulin resistance, and this explains the impaired inhibition of gluconeogenesis in an insulin resistant subject. The pericentral cells, still sensible to insulin signaling, respond to the hormone by increasing *de novo* lipogenesis. Therefore, in NAFLD, *de novo* lipogenesis is still aroused by insulin and it enhances liver fat deposits (Hijmans et al., 2014). Even hyperglycemia activates *de novo* lipogenesis, but through a different pathway, involving the carbohydrate response element binding protein (ChREBP). This can be activated by intermediates of glycolysis such as glucose 6-phosphate or fructose-2,6-biphosphate.

Obesity is strictly related to NAFLD. Adipose tissue is a very active endocrine organ that produces hormones and cytokines. Adiponectin is the major adipose-specific adipokine and it has powerful anti-inflammatory and insulin sensitizer effects. Several studies demonstrated an inverse correlation between adiponectin levels, which in obesity is reduced, and hepatic steatosis, TGs and LDL levels (Shabalala SC et al., 2020; Stern JH et al., 2016). Another adipokine, resistin, has been

involved in glucose and lipid homeostasis, contributing to the development of NAFLD, insulin resistance and inflammation (Adolph et al., 2017). In addition, adipose tissue is also responsible for the production of pro-inflammatory cytokines such as TNF-α and IL-6, that are elevated in NASH compared to simple steatosis.

Neurodegenerative disorders and Alzheimer disease. Metabolic syndrome may be also associated to reduction in the cognitive performance. Indeed, obesity and diabetes exacerbate the risk and outcomes of neurodegenerative disease, such as Alzheimer's Disease (AD), the most common form of dementia. The common overlapping pathways relating obesity, T2DM, and neurodegenerative disorders are retained to be oxidative stress, mitochondrial dysfunction and inflammation (Verdile et al., 2015). They provide a mechanistic link to progressive cognitive decline, which often appears in metabolic syndrome.

The link between obesity and the risk of dementia has been examined in several studies and different hypothesis have been advanced (Anjum et al., 2018; Singh-Manoux et al., 2018). One of the mechanisms by which obesity causes cognitive dysfunction consists in the activation of the cerebral immune system by fatty acids. In obese subjects, the brain uptake and subsequent accumulation of fatty acids is exacerbated. The saturated fatty acids likely act through the toll-like receptor 4 (TLR4) protein, that detects lipopolysaccharides. TLR4 activation leads to the generation of cytokines in astrocytes inducing an inflammatory response in the hypothalamus (Li et al., 2020). Indeed, neuroinflammation is considered as a key player in the pathogenesis of neurodegeneration (Dorothée, 2018). Recent research revealed that proinflammatory pathway of IκB kinase (IKKβ) and downstream nuclear factor-κB (NF-κB) mediates HFD-induced hypothalamic inflammation to cause metabolic syndrome (Purkayastha et al., 2011). It is of note that in addition to being an inflammatory regulator, IKKβ/NF-κB controls cell survival, growth, apoptosis and differentiation in cell-specific manners (Hayden et al., 2006). Consequently, IKKβ/NF-κB-mediated impairment of hypothalamic adult neural stem cells has been proposed as a neurodegenerative mechanism for obesity and related diabetes in HFD-mice (Li et al., 2012). Moreover, chronic low-grade inflammation, observed in obesity, can increase central inflammation because of the entry of immune cells into the brain across the blood-brain barrier (BBB), whose integrity is compromised. Treatment of cultured microglia with sera derived from HFD obese old mice leads to increased microglial activation and oxidative stress (Tucsek et al., 2014). A systemic immune challenge in mice leads to AD-like brain pathology, including deposition of amyloid precursor protein (APP) and its proteolytic fragments and altered Tau phosphorylation. Moreover, systemic inflammation exacerbates AD-like neuropathology in transgenic mice (3xTg-AD), an established mouse model of

AD (Krstic et al., 2012). Also clinical evidence suggest that AD is accompanied by an inflammatory response. In particular, high peripheral concentrations of IL-6, TNF-α, IL-1β, transforming growth factor beta (TGF-β), IL-12 and IL-18 have been observed in Ad patients (Swardfager et al., 2010). Therefore, obesity could cause cognitive dysfunctions by influencing glial activation in the brain.

Type 2 diabetes can affect brain functions. Indeed, magnetic resonance imaging (MRI) results have pointed out a decrease in the hippocampal size in elderly diabetic patients that could be due to reduced neurogenesis and/or elevated neuronal death (Roy et al., 2020). Multiple factors and pathways could play a role in cognitive decline of diabetic patients, in particular in the appearance of hallmarks of AD, such Aβ plaque accumulation and tau protein neurofibrillary tangle formation in the brain (Fig. 5):

(i) Insulin resistance leads to decreased activation of Akt, key protein in multiple cellular processes, such as glucose metabolism and inhibition of GSK3β, one of the kinases that phosphorylate tau protein. Therefore, increased GSK3β activation causes hyperphosphorylation of tau, a component of neurofibrillary tangles found in AD brains (Terwel et al., 2008).

(ii) Insulin-degrading enzyme degrades insulin as well as Aβ peptide. Therefore, hyperinsulinemia sequesters insulin-degrading enzyme away from Aβ, facilitating its accumulation (Ma et al., 2017).

(iii) Accumulation of advanced glycation end products (AGEs), that represents a diabetic complication due to hyperglicemia, in the brain (Chaudhuri et al., 2018). The interaction of AGEs with its receptors, named Receptor for Advanced Glycation Endproducts (RAGE), elicits the formation of ROS, which are also believed to be an early event in AD pathology. Specifically, accumulation of pentosidine and glyceraldehydes-derived pyridinium (GLAP) has been observed in the brain of diabetic rats (Guglielmotto et al., 2012). Pentosidine and GLAP upregulates the expression beta-site APP cleaving enzyme 1 (BACE1), key enzyme in the generation of Aβ, through the binding with RAGE and the consequent activation of NF-κB. AGEs increase also the expression of its receptor, RAGE, which is also a putative receptor for Aβ (Bongarzone et al., 2017). RAGEs have been shown to be upregulated in several cell types in the AD brain. For example, presence of increased numbers of RAGE-immunoreactive microglia (Lue et al., 2001) as well as in astrocytes of AD brain has been reported (Sasaki et al., 2001). Microglia-specific overexpression of RAGE in transgenic AD mice enhances the production of proinflammatory cytokines along with accelerated cognitive decline (Fang et al., 2010).

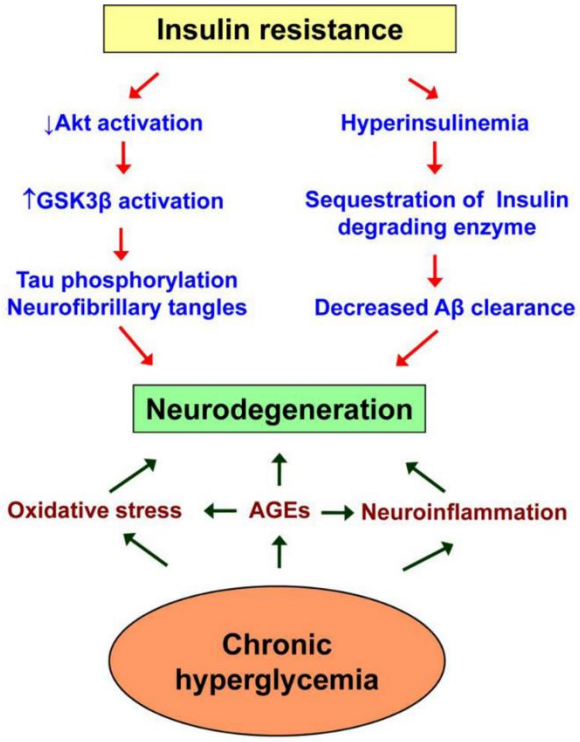

Fig. 5 Pathways of neurodegeneration in the diabetic brain

Glucolipotoxicity is known to cause oxidative stress and mitochondrial injury (Alnahdi et al., 2019). Mitochondrial dysfunction is another critical link between obesity, diabetes, and neurodegeneration. Oxidative stress and mitochondrial dysfunction have been extensively reported both in patients and animal models of AD, diabetes, and obesity. Mitochondrial injury triggers formation of inflammasome (West et al., 2015), a multiprotein cytosolic complex, that can be generated in response to infection, cellular damage, and metabolic dysregulation. Its formation leads to activation of caspase-1 and secretion of the cytokines IL-1β and IL-18 resulting in apoptotic and pyroptotic cell death (Zheng et al., 2020). The inflammatory pathway protects the brain from infection in patients, whereas the formation of sterile inflammasomes in response to cellular stress cause neuronal injury (Kaushal et al., 2015).

1.2 Management of MetS

Management of MetS involves a dual approach that combines lifestyle changes and pharmacological interventions.

Lifestyle modification. As described earlier, MetS results from increased calorie consumption disproportionate to metabolic requirements. Lifestyle modification is imperative in the management of underlying risk factors. Weight reduction and maintenance of ideal body weight are

essential preventive and management strategies. Dietary modification such as low intake of saturated fats, trans fats, cholesterol, sodium and simple sugars can improve dyslipidemia, hyperglycemia and hypertension. Regarding specific dietary patterns, probably the Mediterranean diet, a vegetable fat dietary pattern, is the best strategy to reduce incidence and to lower the prevalence of MetS (Salas-Salvadó et al., 2016). Although the Mediterranean diet has high fat content ranging from 35% to 45% of energy, lipids are represented by unsaturated fatty acids. The Mediterranean diet has been consistently shown to be cardioprotective (Martinez-Gonzalez et al., 2015), suggesting that vegetable high-fat diets are beneficial for cardiovascular health (Mozaffarian & Ludwig DS, 2015). The antioxidant and anti-inflammatory effects of the Mediterranean diet could offer a possible explanation for its beneficial effects on MetS (Di Daniele et al., 2017). Therefore, adoption of the Mediterranean diet may be efficacious for prevention and resolution of MetS.

Exercise increases calorie consumption, aiding weight loss and reducing overall CVD risk: about 30–60 min of moderate intensity exercise can be beneficial for the management of MetS. Regular exercise training combined with adherence to Mediterranean diet intake have been reported to trigger or to augment health protective functions through lipid reduced peroxidation and anti-inflammatory actions, mainly caused by a better microvascular and macrovascular circulation (Casas et al., 2016).

Pharmacotherapy. Pharmacotherapy is another option for the management of MetS. Major pharmacological interventions include treatment of dyslipidaemia with statins, decrease of prothrombotic risk with antiplatelet drugs, use of insulin sensitizers to decrease the risk of diabetes. Indeed, there is no single drug therapy for MetS and currently available pharmacotherapy and associated comorbidities necessitate prolonged use of multiple medications, which is challenging for patients due to polypharmacy and reduced compliance. Thus, there is growing interest in the use of naturally occurring compounds in lowering the risk and progression of MetS though their effect on long-term outcomes and long-term compliance is unknown (Rochani et al., 2017).

Functional foods and nutraceuticals. Functional foods and nutraceuticals have been considered in the treatment of MetS, as they may be effective with a low risk of adverse effects. Functional foods are foods that offer health benefits beyond their nutritional value (Farag et al., 2020). To be classified as “functional”, food must exert its beneficial effects in amounts that can normally be consumed (Wilson et al., 2017). Nutraceuticals comprise any substances (food or parts of a food) providing medical or health benefits, including the prevention and treatment of disease (Wilson et al., 2017). Numerous active food ingredients are in widespread use in traditional

medicine systems such as traditional Chinese medicine and Ayurveda (Yuan et al., 2016). Moreover, higher intake of fruits and vegetables has been linked to improved health status and reduced risk of chronic diseases (Pem & Jeewon, 2015). Mediterranean Diet offers functional foods containing polyphenols, terpenoids, flavonoids, alkaloids, sterols, pigments and unsaturated fatty acids, which play a role in maintaining wellness and in preventing cancer, depression, T2DM, obesity and cognitive decline (Elmaliklis et al., 2019). The main food components responsible for the health effects include: monounsaturated fatty acids such as oleic acid in olive oil, omega-3 polyunsaturated fatty acids (e.g., alpha-linolenic acid) in nuts (Sokoła-Wysoczańska et al., 2018), eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA) in oily fish (Innes & Calder, 2018), flavonoids and antioxidants in fruits and vegetables (Panche et al., 2016) and fibers in cereal and whole-grain foods with a low glycemic index (Călinoiu & Vodnar, 2018).

2. Pistachios

Pistachios have been part of the human diet since prehistoric times and have been consumed by past civilizations because of their nutritional and potential disease-management properties (Hernández-Alonso et al., 2016). Native to the Middle East, the pistachio tree (*Pistacia vera* L., Anacardiaceae family) spread from the Middle East to the Mediterranean, with the nuts becoming a valued delicacy among royalty, travellers, and commoners alike. Since ancient times, pistachio has been used as a folk remedy for a variety of ailments.

2.1 Bioactive components of pistachios

Pistachios are nutrient-dense nuts, containing fiber, unsaturated fatty acids, proteins, minerals, vitamins and a number of beneficial phytochemicals.

Fat content. Pistachios, compared with other nuts, are high in fat, containing 43.4 g fat per 100 g pistachio kernel and consisting of 5.6 g saturated fatty acids (SFA), 13.3 g PUFA and 24.5 g MUFA (Fig. 6). Oleic and linoleic fatty acids, both recognized for their cardiovascular-preventive properties (Perdomo et al., 2015), represent more than 60 % of the total fat content. It is to note that the composition in fatty acids varies depending on the climate in which the plants are grown. For example, cultivars of pistachio nuts grown in hot temperatures (over 25°C) tend to produce a lower amount of palmitic acid, a saturated fat (Satil et al., 2003).

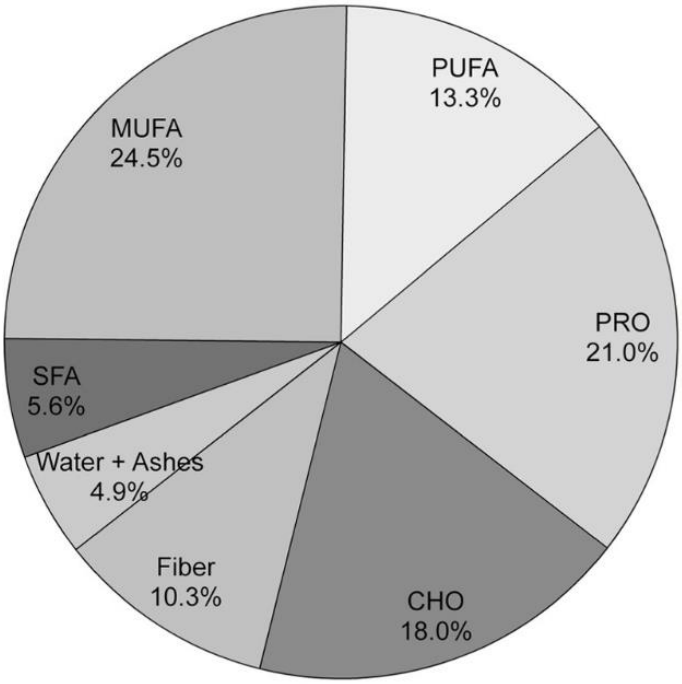

Fig. 6 Macronutrient composition of pistachio nuts

Proteins. Pistachios are a good source of vegetable proteins (about 21% of total weight), with approximately 2% L-arginine (Rodríguez-Bencomo et al., 2015). This amino acid, also present in other nuts, is the precursor to the endogenous vasodilator, nitric oxide (NO). NO is involved not only in the cardiovascular system as a key regulator of vascular tone and, consequently in hypertension and CVD, but also in neurodegenerative disorders due to its pro-oxidant capacity (Förstermann et al., 2017). Therefore, pistachios could play an important protective role in NO-related diseases. Compared with the Food and Agriculture Organization (FAO) and WHO-recommended essential amino acid pattern for an adult, pistachio contain adequate amounts of all essential amino acids (Shivakumar et al., 2020). Pistachios have an essential amino acid/total amino acid ratio of 39.1, higher than other commonly consumed nuts (almonds, walnuts, pecans and hazelnuts). Pistachios also provide a high percentage of branched-chain amino acids (1.599 g leucine, 0.932 g isoleucine and 1.262 g valine per 100 g).

Carbohydrates and fibre. The amount of carbohydrate in pistachios, as in other nuts, is moderately low (about 27.5 %), but pistachios are rich in fiber (10% by weight of insoluble forms and 0.3 % of soluble forms). Fiber content is significant because epidemiological and clinical studies have consistently demonstrated that fiber intake is inversely associated with weight gain (Bozzetto et al., 2018), diabetes (Dahl & Stewart, 2015), CVD (Anderson et al., 2000) and some types of cancer (Chen et al., 2016). Moreover, pistachios have a low glycemic index, which can

contribute to maintaining satiety longer and lowering postprandial blood glucose concentrations (Assaf-Balut et al., 2017).

Vitamins and minerals. Pistachios are rich in Cu, Mg, Mn, vitamin A, vitamin C and B vitamins, with the exception of vitamin B12 (cyanocobalamin), compared with other nuts (Table 6). In particular, pistachios contain relatively high amounts of thiamin (vitamin B1), which is involved in intermediary carbohydrate metabolism. The amount of pyridoxine (vitamin B6) that is involved in the metabolism of amino acids and in the production of niacin is about 1.12 mg/100 g of pistachios. Finally, the amount of folic acid in pistachios provides approximately 25 % of the recommended dietary Aallowances. Among nuts, pistachios also stand out for high vitamin K content, with approximately 13.2 µg/100 g. Of note, a high dietary intake of vitamin K has been associated with a lower risk of several chronic diseases such as T2DM (Ho et al., 2020), cancer and CVD (Simes et al., 2020). They contain also, a relatively high amount of γ-tocopherol, known for its antioxidant properties.

Table 6 Vitamin content of pistachios per 100 g (Dry Roasted)

	Pistachio nuts
Vitamin A, IU	266
Vitamin B_6, mg	1.12
Vitamin B_{12}, mg	0
Vitamin C, mg	3.0
Vitamin D, mg	0
α-Tocopherol, mg	2,17
β-Tocopherol, mg	0,13
γ-Tocopherol, mg	23,42
δ-Tocopherol, mg	0,55
Vitamin K, µg	13,2
Folate, µg	51
Choline, mg	71,4
Betaine, mg	0,8
Thiamine, mg	0,70
Riboflavin, mg	0,23
Niacin, mg	1,37
Pantothenic acid, mg	0,51
Lutein + zeaxanthin, µg	1160
α-Carotene, µg	0
β-Carotene, µg	159

Pistachios are rich in several minerals such as K, Mg, Ca, Cu and Mn. Because of their mineral profile, pistachios could play a beneficial role in blood pressure regulation or in bone-related diseases. Pistachios also contain significant amounts of Zn and Se, minerals with recognized

antioxidant effects that are involved in the prevention of CVD and some types of cancer (Hercberg et al., 2010).

Phenol content. Pistachios, pecans and walnuts are rich sources of phenolic compounds, including anthocyanins, flavonoids, proanthocyanidins, flavonols, isoflavones, flavanones, stilbenes, phenolic acids and hydrolysable tannins, antioxidants with chemopreventive, cardioprotective and vasoprotective properties (Panche et al., 2016). Phenolic compounds may have protective effects against diseases related to free radical overproduction, such as CVD and cancer. A double-blind, randomized, placebo-controlled trial showed that a supplement of 160 mg of anthocyanins, twice daily for 24 weeks in hypercholesterolemic subjects, increased high-density lipoprotein cholesterol and decreased low-density lipoprotein cholesterol concentrations. It also increased the activity of Paraoxonase 1 (PON1), an enzyme able to remove harmful oxidised-lipids and, in turn, to protect against the development of atherosclerosis (Zhu et al., 2014). All phenolic compounds are present in higher amounts in the pistachio skin than in the seed. *Pistacia vera* L. (variety Bronte) skins contain cyanidin-3-O-galactoside (5865mg/g), gallic acid (1453mg/g), catechin (377 mg/g) and eriodictyol-7-O-rutinoside (366 mg/g). Pistachio kernels contain quercetin-3-O-rutinoside (98.1mg/g), genistein (69.1mg/g), genistein-7-O-glucoside (47 mg/g) and daidzein (42.4 mg/g). The total content of flavonoids in the skins is 70.27 mg of catechin equivalents/g of fresh weight, whereas in the seeds, it is only 0.46 mg (Tomaino et al., 2010). Pistachios are the only nut containing anthocyanins and phenolic compounds in the skin. The phenolic compounds are known to bind metals through o-diphenol groups, and, in turn, to inhibit metal-induced lipid oxidation (Dangles & Fenger, 2018).

Carotenoids. Lutein and zeaxanthin are two xanthophyll carotenoids responsible for giving color to pistachio nuts. Raw pistachios contain 1405 mg lutein and zeaxanthin/100 g, about thirteen times more than hazelnuts, which contain only 92 mg %. The bioavailability of carotenoids depends on the source and interaction with other dietary components (Mandalari et al., 2013). Carotenoids have antioxidant properties and they have been associated with a reduced risk of CVD and some types of cancer (Tapiero et al., 2004).

Total phytosterols. Among nuts, pistachios have the highest phytosterol content (214 mg/100 g). It includes stigmasterol, campesterol and β-sitosterol. Phytosterols, structurally similar to cholesterol, have the same basic cyclopentanoperhydrophenanthrene ring structure but differ in the side chain at C24 and/or the position and configuration of unsaturated double bonds and the

optical rotation at chiral carbons. Several studies have demonstrated a dose–response reduction of cholesterol mediated by phytosterols (Cabral & Klein, 2017). Although 500 mg of phytosterols are needed to support the Food and Drug Administration (FDA) health claim, the levels of phytosterols in pistachio nuts may be sufficient to play a synergistic role with unsaturated fatty acids and the low saturated fatty acid levels in maintaining normal cholesterol levels.

2.2 Health benefits of pistachio

The present section deals with the health benefits of the pistachios, as suggested by the nutritional profile.

Pistachio antioxidant potential. ROS are involved in the pathology of different human diseases, thus the antioxidants can be helpful in overcoming oxidative injuries and modulating biological pathways and cell membrane functionality. Pistachios are a rich source of fat-soluble antioxidants and therefore they could have an impact in controlling oxidative stress. Smeriglio and collaborators (2017) evaluated *in vitro* the antioxidant, properties of essential oil from *Pistacia vera* L. variety Bronte by using several antioxidant assays (hydrogen atom transfer and electron transfer-based methods). The pistachio essential oil showed a strong iron chelating activity and it was markedly active against hydroxyl radical, while scarcely active against 2,2-diphenyl-1-picrylhydrazyl (DPPH) radical (Smeriglio A et al., 2017). However, the antioxidant activity of pistachios may be involved in cellular protection, not only in a straightforward manner as a source of antioxidant molecules but also indirectly as a stimulator of the activity of antioxidant enzymes. In fact, the treatment with *Pistacia lentiscus* fruit oil on carrageenan-induced paw edema in rats significantly increases the activities of catalase, superoxide dismutase (SOD), and glutathione peroxidases and significant decreases in the malondialdehyde (MDA) level (Khedir et al., 2016).

The antioxidative efficacy of pistachios has been also shown in a randomised, double-blind, placebo-controlled trial. Patients with active inflammatory bowel disease were randomly allocated in two groups: one receiving natural *Pistacia lentiscus* supplement (2.8 g/day) and the other one receiving placebo for 3 months. Plasma oxidised LDL (oxLDL), oxLDL/ high-density lipoprotein (HDL), and oxLDL/LDL, fell significantly only in patients receiving pistachio supplement, demonstrating that pistachios exhibits favourable effects counteracting oxidative stress (Papada et al., 2018). Since pistachio is a complex natural mixture of fatty acids, monoterpenes, polyphenol, flavonoids, tocopherols, and sterols metabolites, the antioxidant activities may be related to synergistic interaction of both the major and minor components within it.

Pistachios anti-inflammatory potential. Inflammation is a normal protective response, induced by tissue injury or infection, to contrast microorganism and non-self-cell invasion and to remove dead or damaged host cells. In the inflammatory response, there is an increase of permeability of endothelial lining cells and influxes of blood leukocytes into the interstitium, oxidative burst, and release of cytokines. At the same time, there is an induction of the activity of several enzymes (oxygenase, nitric oxide synthase, peroxidase) as well as the arachidonic acid metabolism. In addition to the ability of pistachio to scavenge free radicals, there is also evidence for anti-inflammatory activity. The anti-inflammatory proprieties of pistachio kernels have been analysed both *in vitro* and *in vivo*. *In vitro* experiments, using monocyte/macrophage cell line J774, demonstrated that pre-treatment with pistachio extracts exerted a significant protection against LPS-induced inflammation. It reduced the degradation of NFKB inhibitor-α (IκB-α) and it decreased the TNF-α and IL-1β production in a dose-dependent way (Paterniti et al., 2017). *In vivo* experiments showed that pistachio polyphenols reduced histological damage, consisting in neutrophil infiltration and nitrotyrosine formation, induced by carrageenan injection in rat paw (Paterniti et al., 2017).

The effects of pistachio consumption on markers of inflammation have been examined also in humans, but the results are inconsistent. A significant reduction in Il-6 A, with no change in TNF-α and CRP, was observed in young healthy males after 4 weeks of pistachio diet (Sari et al., 2010). In T2DM adults after 4 weeks of nutritionally-adequate diet with pistachios no change in inflammatory markers were observed (Sauder et al., 2015). However, although pistachio consumption for 4 months did not affect circulating inflammatory markers in a sample of adults with pre-diabetes, it reduced lymphocyte gene expression of Il-6 (Hernández-Alonso et al., 2014). Moreover, a 24-wk randomized clinical trial with a greater number of participants, showed that a longer intervention period with use of pistachios could reduce inflammatory markers in Asian subjects MetS (Gulati et al., 2014). The variability in the results obtained could depend on the duration of the treatment, participant demographics, and markers of inflammation evaluated, thus it difficult to draw definitive conclusions about the role of regular pistachio intake on the inflammation.

Pistachio, satiety and body weight. Because nuts are energy-dense foods with a high fat content, one of the main concerns regarding the regular consumption of nuts in a worldwide pandemic of overweight and obesity is that nuts are believed to be fattening. To date, however, epidemiological studies have failed to find any association between nut or pistachio consumption and either weight gain or an increased risk of obesity. A recent study showed that daily intake of 44 g pistachios for 12 weeks improved dietary profile without affecting body weight or composition in

healthy women (Fantino et al., 2020). The additional calories provided by the pistachios induced satiety and sufficient adjustment of intake to prevent body weight changes. Consistently, another study on French women showed that pistachio consumption had no impact on body weight. Indeed, a daily pistachio snack for a month did not affect body weight or composition but it improved micronutrient intake (Carughi et al., 2019). The effect of pistachio consumption has been also examined also in overweight/obese adults. This randomized controlled study enrolled non-diabetic overweight/obese adults (n = 100) assigned to a 4-month behavioural weight loss intervention only (control group) or also prescribed 42 g/day of pistachios (pistachio group). The results showed that the regular consumption of pistachios was associated with a comparable degree of weight loss, and similar reductions in BMI and waist circumference, compared to controls. Additionally, regular pistachio consumption was associated with healthful shifts in dietary intake and food choices, including increased dietary fibre, decreased consumption of sweets, and a more favourable ratio of PUFA and MUFA/saturated fatty acids (Rock et al., 2020). These findings may be explained by pistachio content in fibre, protein, and unsaturated fatty acids and by their crunchy physical structure, which may induce satiety and therefore reduce subsequent food intake. It has been speculated that various mechanical/chemical sensory systems, activated by mastication, may modify appetitive sensations (McCrickerd et al., 2016). In addition, the MUFA and PUFA that nuts contain can induce higher thermogenic effect than saturated fatty acids (Vázquez Cisneros et al., 2019), leading to less fat accumulation (Saito et al., 2020).

Pistachio and cardiovascular disease. The impact of pistachio consumption as a possible protective factor in the cardiovascular has been also taken into account. Significant effects in preventing hypertriglyceridemia and hypercholesterolemia have been observed in rats with metabolic syndrome, that received kernel oils of wild pistachio (2 ml/kg/day) for 10 weeks (Jamshidi et al., 2018). Another study investigated the pistachio effect on atherosclerosis. Rabbits received atherogenic diet supplemented with methanolic or cyclohexane extracts of the *Pistacia vera* nut for 3 months. Pistachio extracts were beneficial on HDL-, LDL-cholesterol and aortic intimal thickness. The methanolic extract additionally presented an antioxidant effect and it significantly decreased aortic surface lesions (Marinou et al., 2010), suggesting that pistachio dietary inclusion is also potentially beneficial in atherosclerosis management. Moreover, a randomized study assessed the effect of pistachio consumption on blood pressure, systemic hemodynamics, and heart rate variability in adults with well controlled type 2 diabetes. Participants consumed a low fat control diet (27% fat) containing low fat/high carbohydrate snacks and a moderate fat diet (33% fat) containing pistachios (20% of total energy) for 4 weeks each. The

pistachio diet significantly reduced total peripheral resistance, increased cardiac output, and improved some measures of heart rate variability. Systolic blood pressure was significantly reduced following the pistachio diet, with the greatest reduction observed during sleep (Sauder et al., 2014). Another study showed that the intake of 10% of energy in the form of pistachios for 1 month significantly reduced systolic blood pressure and made no difference in diastolic blood pressure compared with the control nut-free group (West et al., 2012). Although these evidence support daily pistachio consumption as beneficial factor against cardiovascular risk factors, others clinical trials need to be carried out to establish the real potential effects of pistachio consumption on the prevention of cardiovascular events.

Pistachio and glucose homeostasis. Although pistachios have relatively high content of carbohydrates (18% w/w), the pistachio consumption is not associated to harmful effects in subjects with glucose metabolism impairment. Pistachios have a low glycaemic index, suggesting that they may reduce postprandial glycaemia and insulinemia and therefore contribute to reducing the T2DM risk. Indeed, Parham and collaborator observed a marked decrease in HbA1c and fasting blood glucose concentrations in patients with T2DM after 12 weeks of pistachio supplementation (25 g twice a day), but no effect on insulin resistance (Parham et al., 2014). These observations were confirmed by other researchers, showing that the addition of pistachios to a meal containing foods rich in carbohydrates with a high glycaemic index such us white bread, reduced postprandial glycaemia, increased glucagon-like-peptide levels, without affecting postprandial insulin levels (Kendall et al., 2014). Nevertheless, another study did not find significant changes in insulin concentrations and in fasting plasma glucose during the pistachio-enriched diet period in non-diabetic subjects with metabolic syndrome (Wang et al., 2012). Results from a randomized controlled trial showed a reduction of gestational diabetes mellitus incidence in normoglycemic pregnant women consuming a diet supplemented with extra virgin olive oil and pistachios (Assaf-Balut et al., 2017). Therefore, further experimental evidences are need in order to consider pistachios as a beneficial supplement in glucose dysmetabolism.

In conclusion, although various studies have addressed the potential beneficial effects of adding pistachios to ordinary diets, clear results have not obtained yet. Therefore, we have undertaken the present research in the attempt to clarify the controversial aspects and to provide a more complete picture by examining unexplored obesity-related disorders, including hepatic steatosis and neurodegeneration.

AIM OF THE RESEARCH

The present research was undertaken to investigate the potential effects of regular intake of pistachio on the obesity-related metabolic dysfunctions. To this purpose, we used an animal model with diet-induced obesity (HFD mouse), that develops obesity and progressive metabolic dysfunctions similar to human metabolic syndrome including neurodegeneration. It is considered a very helpful model to analyse the potential effects of different treatments on obesity-related disorders (Nuzzo et al., 2015; Collins et al., 2004).

In detail, we investigated:

- The impact of pistachio intake on different aspects of metabolic syndrome, such as glucose and lipid homeostasis, hepatic steatosis and adiposity.
- The mechanism of action responsible of the effects observed.

- The impact of pistachio intake on visceral and systemic inflammation.
- The influence of pistachio intake on intestinal microbiome composition.

- The impact of pistachio intake on neurodegeneration and brain oxidative stress.

Mice, separated in groups, were fed differently for 16 weeks, as it follows: 1. Lean group, fed a standard diet (STD) 2. HFD group, fed a HFD (untreated HFD mice) 3. HFD-P group, fed a HFD supplemented with pistachio. HFD-P was custom prepared by Mucedola S.r.l (PF4215/C; R&S 34/16) by substituting 20% of HFD caloric intake with pistachio (180 g/Kg HFD). At the end of the 16th weeks, the animals were sacrificed and different organs and tissues were opportunely explanted. Different parameters were evaluated and compared among three animal groups.

I° ARTICLE

PISTACHIO CONSUMPTION PREVENTS LIPID DYSMETABOLISM BY REDUCING THE LIPID METABOLIZING GENE EXPRESSION IN DIET-INDUCED OBESE MICE

Disclosure

The results included in this paper have been published in the Journal *Nutrients* (Terzo et al., 2018a). Some results have been published in abstract form (Terzo et al., 2018 a; Terzo et al., 2018 b; Terzo et al., 2018 c).

Summary

The experiments described in this study examined if pistachio consumption is able to prevent hyperglycemia, dyslipidemia, hepatic steatosis, and adipose tissue morphological alterations caused by high fat diet (HFD) in the mouse. Moreover, the impact of pistachio intake on the mRNA expression of peroxisome proliferator-activated receptor γ (PPAR-γ), fatty acid transport proteins (FAT-P), fatty acid synthase (FAS), stearoyl-CoA desaturase (SCD1), and sterol regulatory element-binding transcription factor-1c (SREBP-1c) in liver and adipose tissue was also analysed. No change in body weight, food intake, and glycaemia was observed between mice consuming pistachios (HFD-P) and HFD mice. Pistachio intake was able to prevent HFD-induced hypertriglyceridemia. Cholesterol plasma levels, steatosis grading, body fat mass, and adipocyte size were significantly lower in HFD-P group compared to HFD. Pistachio-diet was able to prevent HFD-induced overexpression of PPAR-γ, FAS, and SCD1 in the liver and SREBP-1c, PPAR-γ, and FAT-P in adipose tissue. The present study shows that pistachio consumption is able to prevent obesity-related dysfunctions by positively modulating the expression of genes linked to lipid metabolism.

Introduction

Metabolic syndrome (MetS) is a cluster of conditions that increase the risk of cardiovascular diseases and diabetes mellitus because it is characterized by obesity, hyperglycaemia, insulin resistance, hypertension, and dyslipidaemia. The pathogenesis of MetS is a complex issue involving genetic, environmental, and dietary factors (Phillips, 2013). It is well known that a high-fat-diet

(HFD) and excessive nutrient intake result not only in adipose tissue (AT) triglyceride accumulation, with consequent adipocyte hypertrophy and pro-inflammatory cytokines release, but also in ectopic fat deposition. Excessive hepatic lipids can lead to steatosis, the initial stage of non-alcoholic fatty liver disease (NAFLD). NAFLD is strictly linked to atherogenic dyslipidaemia and diabetes and it is considered to be a hepatic component of MetS (Suárez et al., 2017). Several metabolic and signaling pathways are involved in perpetrating the obesity-metabolic disturbances observed in the liver and AT. In particular, recent studies reported that hepatic or adipose tissue genes concerning synthesis and transport of fatty acids, including fatty acid transport proteins (FAT-P), sterol regulatory element-binding transcription factor-1c (SREBP-1c), stearoyl-CoA desaturase (SCD1), fatty acid synthase (FAS) and peroxisome proliferator-activated receptor γ (PPAR-γ), are upregulated in HFD rodents (Huang et al., 2016; Zhuang et al, 2017).

Diet-based strategies, such as foods with hypolipidemic, anti-oxidant and anti-inflammatory properties, are recommended when dealing with MetS.

Natural remedies are currently drawing attention as therapeutic or protective agents in treating MetS because natural plant compounds seem to improve obesity-related dysfunctions (Kim et al, 2018; Lee et al., 2015).

Health benefits of regular nut consumption (mainly pistachios, almonds, and walnuts) have been well-documented in studies both on animals and humans (Ros, 2010; Terzo et al., 2019; Vadivel et al., 2012;). Daily nut consumption can improve dysmetabolic conditions such as obesity, type 2 diabetes mellitus (T2DM), and related cardiovascular diseases (Tan et al., 2014; Askari et al., 2013; Del Gobbo et al., 2015).

Compared to other nuts, pistachios have a higher amount of monounsaturated fatty acid (MUFA) and polyunsaturated fatty acid (PUFA) (Terzo et al., 2019; Hernández-Alonso et al., 2016; D'Evoli et al., 2015). They are rich in phytosterols (stigmasterol and campesterol), lutein (xanthophyll carotenoid), and polyphenols (resveratrol and catechins) (Terzo et al., 2019; Tan et al., 2014; Askari et al., 2013; Del Gobbo et al., 2015; Hernández-Alonso et al., 2016; D'Evoli et al., 2015; Herbello-Hermelo et al., 2018). These substances are known for their anti-inflammatory and antioxidant actions (Terzo et al., 2019; Gentile et al., 2012; Vaidya et al., 2017; Vilahur et al., 2019). Therefore, regular pistachio intake could have higher beneficial potential than other nuts. Although pistachios are commonly considered a fattening food, on account of their high fat content, different studies have shown that the addition of pistachios to ordinary diets did not induce weight gain (Vadivel et al., 2012; Terzo et al., 2019; Wang et al., 2012). The advantageous effects of regular pistachio intake on lipid profile remain controversial. In fact, previous studies on humans and animals reported decreases or no effect on low-density lipoprotein (LDL) after pistachio

consumption (Askari et al., 2013; Del Gobbo et al., 2015; Wang et al., 2012; Gebauer et al., 2008; Aldemir et al., 2011; Edwards et al., 1999; Kocyigit et al., 2006; Sheridan et al., 2007; Hernández-Alonso et al., 2014).

Nevertheless, the potential beneficial properties of pistachio consumption on other obesity-related dysfunctions, such as hepatic steatosis and adipose tissue morphological alterations, have not been explored yet.

The purpose of the present study was to investigate if pistachio consumption is able to prevent obesity-related metabolic dysfunctions, such as hyperglycaemia, dyslipidaemia, hepatic steatosis, and AT morphological alterations, in HFD mice. In addition, the effects of regular pistachio intake on the expression of genes linked to fatty acid synthesis and lipid uptake, such as SREBP-1c, PPAR-γ, SCD1, FAS, and FAT-P, were analysed in liver and adipose tissue to explore the mechanism of action responsible for the beneficial effects.

Materials and Methods

Animals

The procedures were performed in accordance with the Italian legislative decree N° 26/2014, and the European directive 2010/63/UE.

The experimental protocols were approved by the animal welfare committee of the Istituto Zooprofilattico Sperimentale della Sicilia "A. Mirri" (Palermo, Italy) and authorized by the Ministry of Health (Rome, Italy; Authorization Number 349/2016-PR).

Four-week old male C57BL/6J (B6) mice, purchased from Harlan Laboratories (San Pietro al Natisone-Udine, Italy) were housed under standard conditions of light (12 h light:12 h darkness cycle) and temperature (22–24 ◦C), with free access to water and food. Mice were allowed to acclimate for 1 week prior to the implementation of the special diets.

Mice were randomly divided into three groups: (1) Lean group: control animals fed with standard diet (STD; 70% of energy as carbohydrates, 20% protein, and 10% fat; 4RF25 Mucedola, Milan, Italy) for 16 weeks; (2) High-fat diet (HFD) group: obese animals fed with HFD (60% of energy as fat, 20% protein, and 20% carbohydrates; PF4215, Mucedola, Milan, Italy) for 16 weeks. The HFD group was used as a control of obesity-related dysfunctions because these animals, consequent to a HFD, develop obesity, hyperglycaemia (Baldassano et al., 2013; Baldassano et al., 2015), hepatic steatosis (Amato et al., 2017), atherosclerosis (Awień et al., 2004), and neurodegeneration (Nuzzo et al., 2015). (3) HFD-P group: obese animals fed with a HFD supplemented with pistachio (HFD-P) for 16 weeks.

HFD-P was custom designed and prepared by Mucedola S.R.L (PF4215/C; R&S 34/16). It was obtained by substituting 20% of the caloric intake from HFD with pistachio (180 g/Kg of HFD). The composition of the different diets are provided in Table 1. Mineral and vitamin mix formulas are shown in Tables 2 and 3. Pistachio nuts belong to *Pistacia vera* L. species and were purchased by Pistachio Valle del Platani Association and Pistacchio di Raffadali (Agrigento-AG, Sicily). Previous analysis of the fat content in this Sicilian cultivar highlighted a very high quantity of monounsaturated and polyunsaturated fatty acids (70% oleic acid, 1% palmitoleic acid, and 18% of linoleic fatty acid) (Terzo et al., 2019). The differences in mono- and polyunsaturated fatty acids between HFD and HFD-P are reported in Table 4.

Table 1. Composition and energy densities of STD, HFD and HFD-P.

Ingredient (g/kg)	**STD**	**HFD**	**HFD-P**
Acid Casein 741	200	265.00	210.00
L-Cystine	2.8	4	4
Maltodextrine-0032	33.2	160	125.5
Sucrose	300	90	100
Cellulose (Arbocel)	50	65.5	50
Soybean oil	25	30	30
Lard	19	220	135
Vitamin mix AIN-93-VX-PF2439	10	21	21
Mineral mix AIN-93G-MX-PF2348	45	48	48
Choline bitartrate	1.9	3	3
Calcium Phosphate dibasic	13	3.4	3.4
Pistachio	-	-	180
Total Energy, Kcal/g	3.5	6	6
Protein, %	20	20	20
Carbohydrate, %	70	20	20
Fat, %	10	60	60

Abbreviations are: STD, Standard diet. HFD, high fat diet. HFD-P, HFD supplemented with pistachio. This study used 4RF25, PF4051/D and PF4215/C-R&S34/16 diets (Mucedola s.r.l.) as STD, HFD and HFD-P, respectively. Composition of these diets is from the Mucedola Web site. The mineral and vitamin mix formulas are shown in Tables 2 and Table 3, respectively.

Table 2. Composition of mineral mix in STD, HFD and HFD-P.

PMIX AIN-93G-MX	
Ingredient (mg/kg)	4800 g/100 kg
Calcium carbonate	6854,40
Potassium phosphate monobasic	2700,10
Potassium citrate tribasic monohydrate	1223,08
Sodium chloride	1395,94
Magnesium oxide	703,34
Potassium sulfate	1004,32
Chromium K sulfate	1,37
Cupric carbonate	8,26
Sodium fluoride	1,38
Potassium iodate	0,28
Ferric citrate	55,27
Manganese carbonate	14,45
Ammonium molybdate	0,21
Basic nickel carbonate	0,15
Lithium chloride	0,14
Boric acid	0,68
Ammonium metavanadate	0,14
Sodium metasilicate	6,88
Zinc carbonate	45,94
Sodium selenite	0,21

Table 3. Composition of vitamin mix in STD, HFD and HFD-P.

PMIX AIN-93G-MX	
Ingredient (mg/kg)	2100 g/100 kg
Vit. K1 phylloquinone 97-103%	1,58 mg/Kg
Nicotinic acid 99,5-100,5%	63,0 mg/Kg
Calcium pantothenate 98-101%	33,6 mg/Kg
Vit. A palmitate 250	8400 I.U./Kg
Biotin 97,5-100,5%	0,42 mg/Kg
Piridoxine 99-101%	14,70 mg/Kg
Riboflavin 97-103%	12,60 mg/Kg
Thiamine 98,5-101%	12,60 mg/Kg
Vit. D3 Cholecalciferol, 500	2100 I.U./Kg
Cyanocobalamin > 0,1%	0,053 mg/Kg
Folic Acid 96-102%	4,16 mg/Kg
α-tocopheryl acetate, 500 IU/g	157,50 mg/Kg

Table 4. Fatty acids composition in HFD and HFD-P.

Fatty Acids (g/kg)	**HFD**	**HFD-P**
total saturated	92.5	71.12
total monounsaturated	117.5	136.65
total polyunsaturated	40	42.23

Abbreviations are: HFD, high fat diet; HFD-P, HFD supplemented with pistachio.

Biochemical Analysis

In euthanized mice, blood was drawn by cardiac puncture and immediately transferred into chilled tubes containing a final concentration of 1 mg/mL ethylenediaminetetraacetic acid (EDTA). Then, the samples were centrifuged at 3000 rpm for 10 min, and the obtained plasma was stored at −80 ◦C until analysis. Aspartate transaminase (AST) and alanine transaminase (ALT) concentrations were measured using the ILAB 600 Analyzer (Instrumentation Laboratory, Bedford, Massachusetts).

Micro-Computed X-Ray Tomography

Micro-computed tomography (micro-CT) scans were performed to assess the volumes of the visceral adipose tissue (VAT) and subcutaneous adipose tissue (SAT) depots.

Four mice from each group were randomly selected and anesthetized with 5% isoflurane. Transverse micro-CT images of the abdomen from L1 to L5 were obtained by the micro-CT scanner Quantum FX µCT (Perkin-Elmer, Hopkinton, MA, USA). Voltage was set at 50 kV and current was set at 200 µA and the images were captured over a 4.5 min interval. Analysis of micro-CT images was conducted with AnalyzePro software (AnalyzeDirect, Overland Park, KS, USA). Visceral and subcutaneous adipose tissue were segmented in the sagittal plane and tissue volumes were expressed relative to body mass (Donato et al., 2014). Experimental data from micro-CT were provided by ATeN Center—Università di Palermo.

Histological Analysis

For the microscopic examination of hepatic and adipose tissue morphology, liver and visceral adipose tissue (including epididymal and retroperitoneal adipose tissue) were fixed in 4% buffered formalin for 24 h. Then, the tissues were dehydrated in alcohol and embedded in paraffin. Paraffin histological sections (5 µm thick) were stained with hematoxylin and eosin and observed using an optical microscope (Leica DMLB, Meyer instruments, Houston, Texas) connected to a high-resolution camera (DS-Fi1, Nikon, Florence, Italy). Grading of steatosis was determined by analyzing the morphology and percentage of lipid vesicles in hepatocytes by an experimenter blinded to treatment conditions. The steatosis was defined as absent, light, moderate, or severe when ≤1%, 30%, 30–60%, or ≥60% of the hepatocytes were respectively involved (Liang et al., 2014). The size of adipocytes was measured according to the cell diameter (Ghorbani et al., 2010). Twenty-thirty adipocytes were measured in different randomly selected optical fields.

Quantification of Hepatic Lipids

Total liver lipids were extracted using a protocol adapted from Folch et al. (Folch et al., 2957). Briefly, the samples were homogenized in ice-cold chloroform: methanol (2:1) solution for 1 min. The homogenate was centrifuged to recover the liquid phase. The solvent was washed with one-quarter of total volume of 0.9% NaCl solution and vortexed vigorously for 30 s. The mixtures were centrifuged at 2000× g for 5 min to separate the two phases. The lower phase containing lipids was evaporated under vacuum in a rotary evaporator. The weight difference between the starting empty tube and the tube containing the dried lipids was the lipid amount.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA was extracted from liver and visceral adipose tissue using the RNeasy plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The extraction from adipose tissue was performed after a preliminary step of lysis using Triazol. Two nanograms of total RNA were used for cDNA synthesis with High Capacity cDNA Reverse Transcription (Applied Biosystems, MA, USA). The target cDNA was amplified using genetic-specific primers, as listed in Table 5. The amplification cycles included denaturation at 95 ◦C for 10 s, annealing at 60 ◦C for 15 s, and elongation at 72 ◦C for 15 s. After 35 cycles, the PCR products were separated by electrophoresis on a 1.8% agarose gel for 45 min at 85 V. The gels were stained with 1 mg/mL ethidium bromide and visualized with ultraviolet (UV) light using E-Gel GelCapture (Thermo Fisher Scientific, Monza, Italy), and the expression levels of the gene targets, normalized to the endogenous reference (β-actin), were analyzed using E-Gel GelQuant Express Analysis Software (Thermo Fisher Scientific, Monza, Italy).

Table 5. Oligonucleotide sequence of primers for RT-PCR.

Gene	Forward primer	Reverse primer	Size (bp)
FAS	5′-TACTTTGTGGCCTTCTCCTCTGTAA-3′	5′-CTTCCACACCCATGAGCGAGTCCAGGCCGA-3′	445
SCD1	5′-GCCAGACCGGGCTGAACACC-3′	5′-GGCCTCCCAAGTGCAGCAGG-3′	397
SREBP-1c	5′-GGAGACATCGCAAACAAGC-3′	5′-GGTAGACAACAGCCGCATC-3′	273
PPAR-ɣ	5′-GGGCTGAGGAGAAGTCACAC-3′	5′-TCAGTGGTTCACCGCTTCTT-3′	142
FAT-P	5′-CGCCGATGTGCTCTATGACT-3′	5′-ACACAGTCATCCCAGAAGCG-3′	138
β-actin	5'- GGATCCCCGCCCTAGGCACCAGGGT-3'	5'-GGAATTCGGCTGGGGTGTTGAAGGTCTCAAA-3'	289

Statistical Analyses

Results are shown as means ± the standard error of the mean (S.E.M.). The letter n indicates the number of animals. Statistical analyses were performed using Prism Version 6.0 Software (Graph Pad Software, Inc., San Diego, CA, USA). The comparison between the groups was performed by ANOVA followed by Bonferroni's post-test. A p-value ≤ 0.05 was considered statistically significant.

Results

Effect of Pistachio Consumption on Metabolic Parameters

After 16 weeks on diet, HFD mice exhibited a significant increase in body weight in comparison with the STD group. Similarly, mice fed with HFD-P were heavier than the lean group but with a mean body weight similar to HFD animals (Figure 1A). No difference in the daily food intake was observed among the three different groups (Figure 1B). Moreover, HFD mice showed fasting glycaemia, triglyceride, and cholesterol plasma levels higher than the STD group (Figure 1C, D) confirming an impairment of glucose and lipid metabolism (Baldassano S et al., 2015; Baldassano S et al., 2016a; Baldassano et al., 2016b). Pistachio consumption did not prevent HFD-induced hyperglycemia (Figure 1C). On the contrary, triglyceride and cholesterol concentrations were significantly reduced in HFD-P mice in comparison with untreated obese mice, although these values were higher than the STD group (Figure 1D).

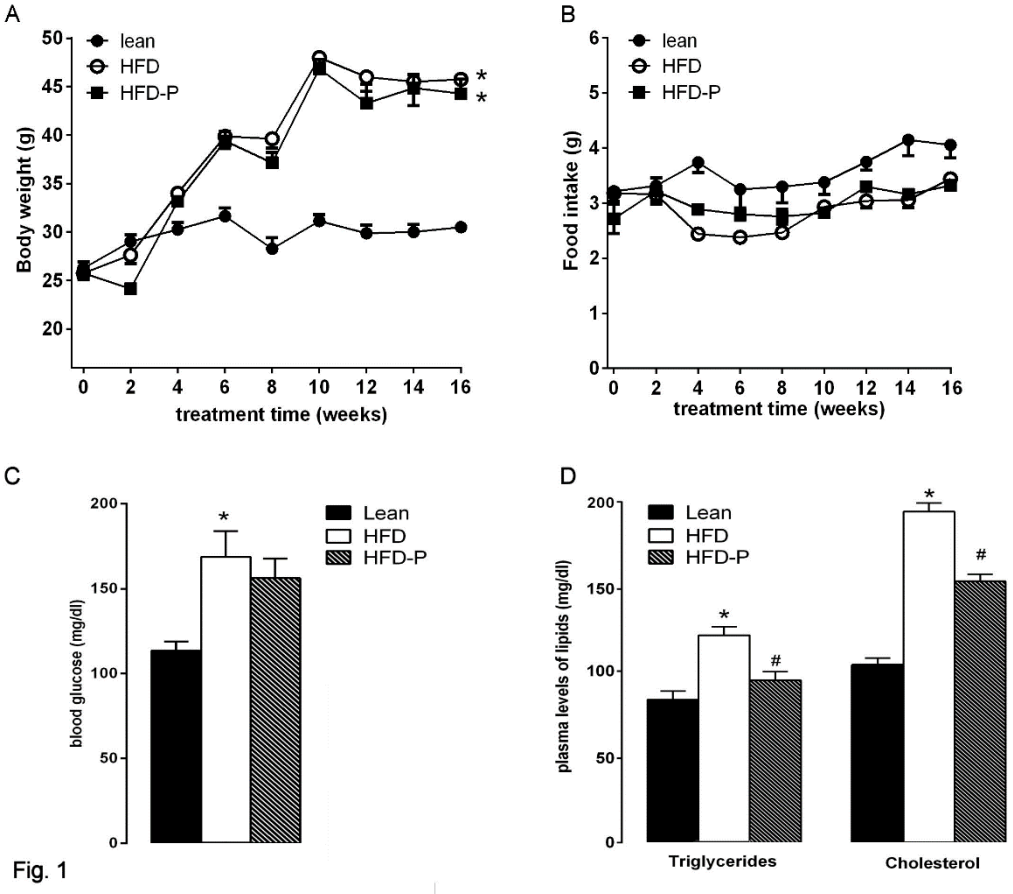

Figure 1. Effects of pistachio consumption on metabolic parameters. Pistachio consumption prevents high-fat diet (HFD)-induced ipertryglyceridemia and hypercholesterolemia. Body weight (A), food intake (B), fasting glycaemia (C) and plasma lipid levels (D) in lean, HFD and HFD-P mice. Data are the means ± S.E.M. (n = 8/group). * $p < 0.05$ *vs* lean; # $p < 0.05$ *vs* HFD.

Pistachio consumption and liver steatosis

The liver samples from STD mice showed normal lobular architecture with absence of steatosis (Figure 2A). After 16 weeks of HFD, the obese mouse liver showed deranged structure of hepatic parenchyma with diffuse fatty infiltration corresponding to moderate steatosis (Figure 2B). In according to the high accumulation of fat droplets, the hepatic lipid content, as well as the absolute and relative (%) liver weight and plasma AST and ALT concentrations were higher than lean group (Figure 2 D-G). Pistachio consumption prevented HFD-induced liver injury; in fact, HFD-P liver showed light steatosis with small lipid inclusion into hepatocytes (Figure 2C). In agreement with the morphology improvements, a significant decrease in the liver fat and weight, ratio liver weight/body weight and serum alanine aminotransferase (ALT) and aspartate aminotransferase (AST) was observed in HFD-P animals in comparison with HFD group (Figure 2D-G).

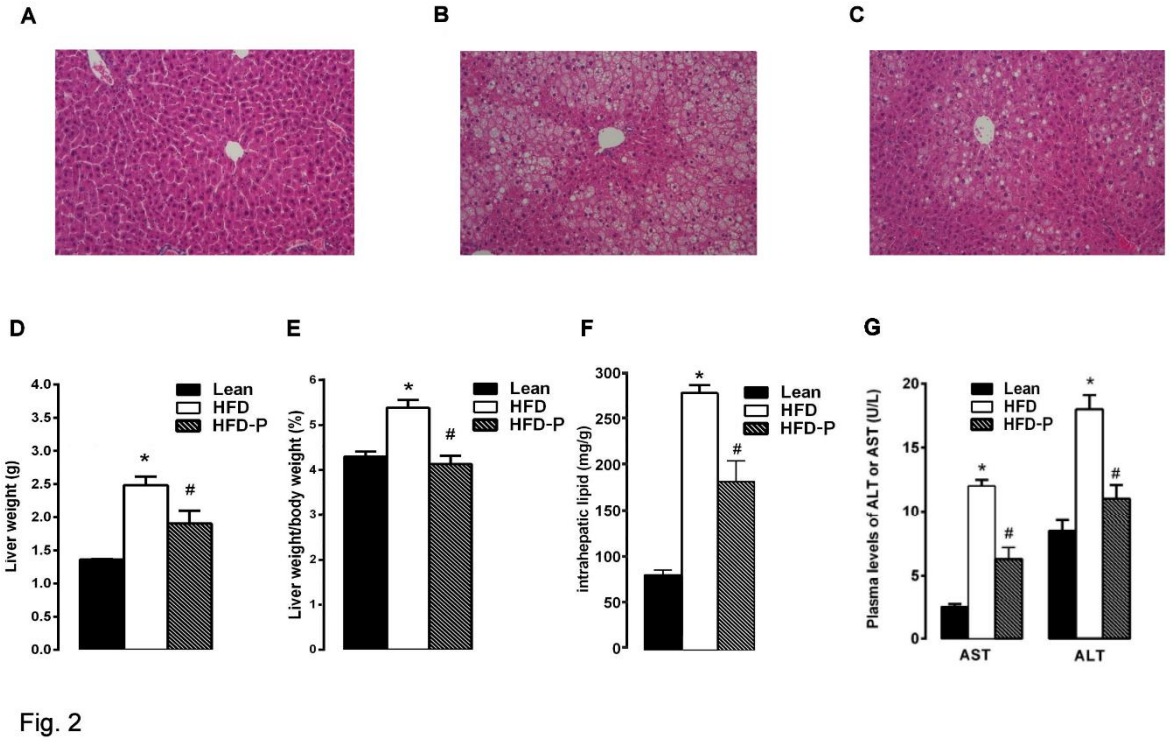

Fig. 2

Figure 2. Pistachio consumption and liver steatosis. Pistachio diet prevents hepatic steatosis development in obese mice. Histological cross-sections of liver from lean (A), HFD (B) and HFD-P mice (C). Hematoxylin and eosin stain. Original magnification: X 200. Liver weight (D), ratio of liver weight/body weight (E), intrahepatic lipid content (F), plasma levels of AST and ALT (G) in lean, HFD and HFD-P mice. Data are the means ± S.E.M. (n = 8/group). * $p < 0.05$ *vs* lean; # $p < 0.05$ *vs* HFD.

Pistachio consumption and HFD-induced adipose tissue alterations

Micro-computed tomography analysis revealed that the visceral and subcutaneous depots were significantly increased in HFD mice compared to lean animals. Interestingly, HFD-P mice

showed a significant reduction of VAT volume/body mass ratio and a significantly increased SAT volume/body mass ratio in comparison with obese animals (Figure 3A-B).

Moreover, histological examination of HFD-VAT revealed a significant increase in adipocyte size, adipose tissue weight and fat index when compared to the lean group. Once more, HFD-P mice showed significantly reduced adipose tissue weight, fat index and adipocyte size in comparison with HFD mice, although the values were higher than STD group (Figure 3 C-H).

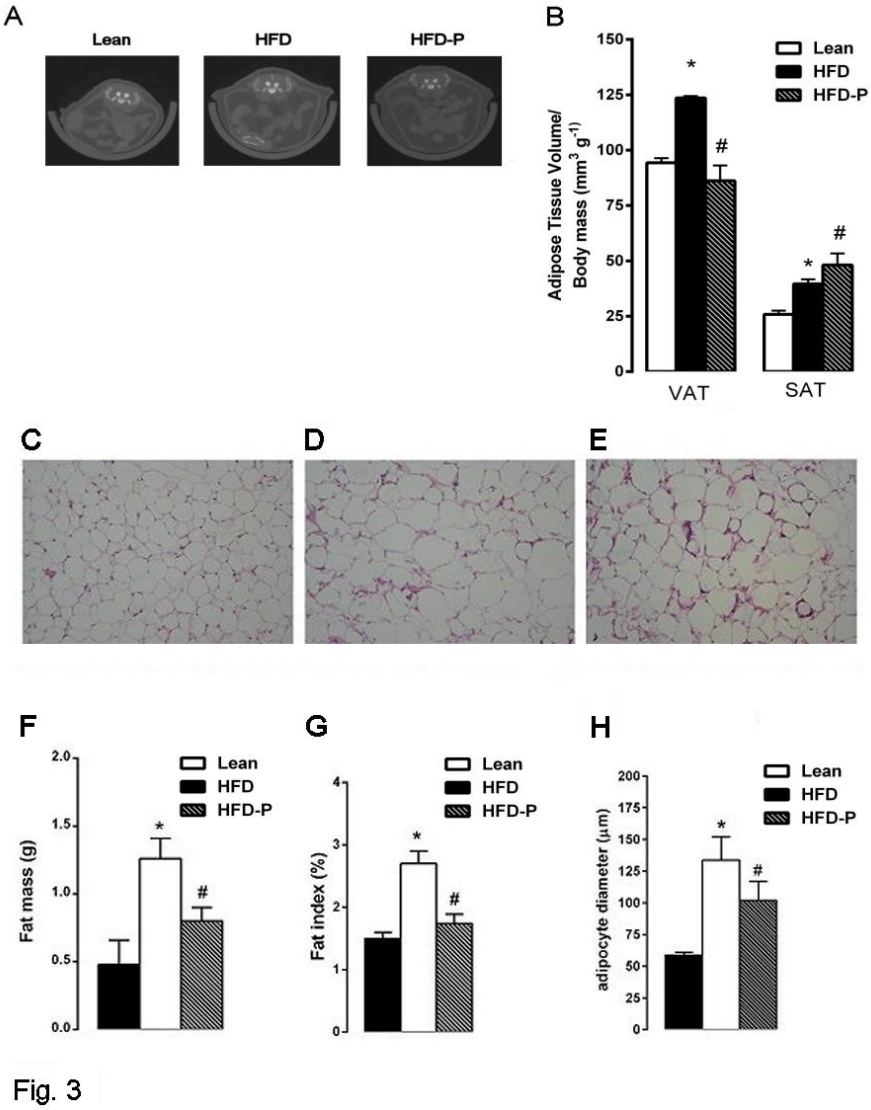

Fig. 3

Figure 3. Pistachio consumption and HFD-induced adipose tissue alterations. Pistachio diet prevents visceral fat accumulation. Transverse micro-CT images of the abdomen (A) and visceral (VAT) and subcutaneous (SAT) adipose tissue volume in lean, HFD and HFD-P mice. Histological cross-sections of VAT from Lean (C), HFD (D) and HFD-P mice (E). Hematoxylin and eosin stain. Original magnification: X 200. VAT weight (F), VAT weight normalized to body weight (fat index) (G), and adipocyte diameter (H) in lean, HFD and HFD-P mice. Data are the means ± S.E.M. (n = 8/group). * $p < 0.05$ vs lean; # $p < 0.05$ *vs* HFD.

Pistachio consumption on lipid metabolism-related genes expression

SREBP-1c, PPAR-ɣ, FAT-P, FAS and SCD1 mRNA levels were significantly higher in HFD liver and adipose tissue compared to the gene expression levels observed in lean mice. On the

contrary, pistachio-diet significantly normalized PPAR-ɣ, FAS and SCD1 gene expression changes in liver and SREBP-1c, PPAR-ɣ and FAT-P gene expression in adipose tissue, compared with the obese group (Figure 4A-D).

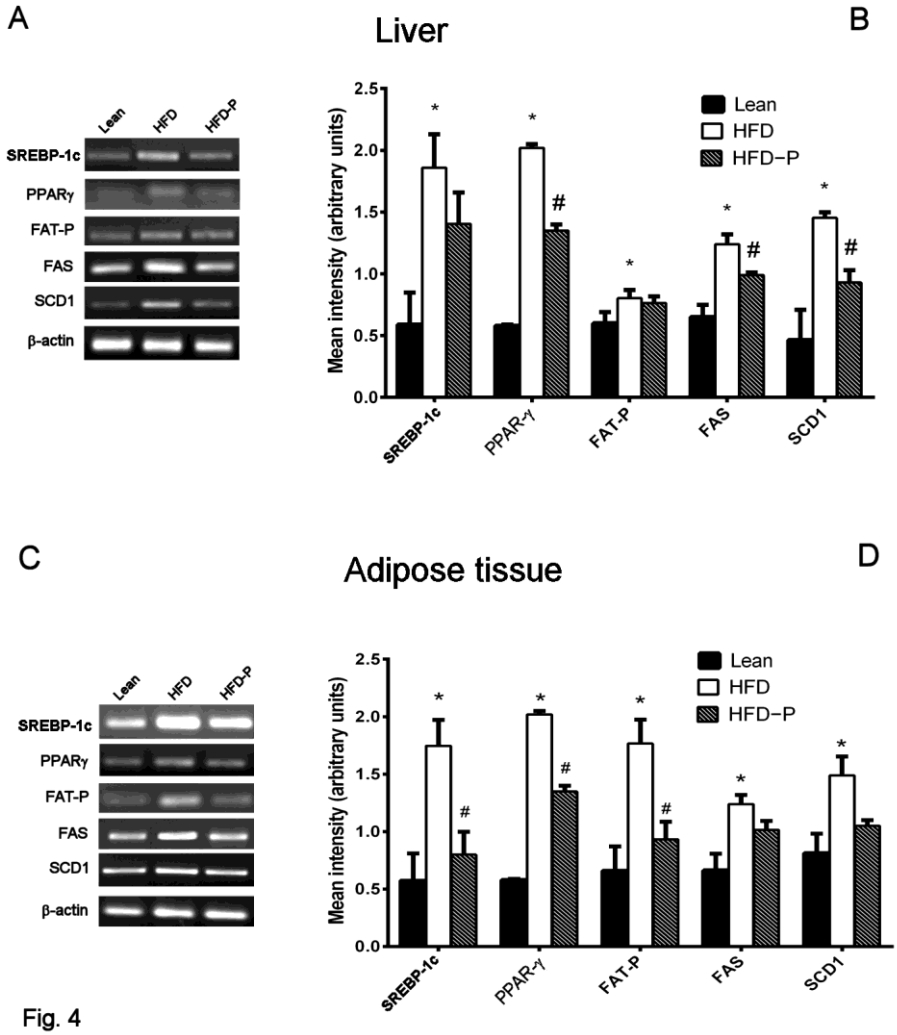

Fig. 4

Figure 4. Pistachio consumption on lipid metabolism-related genes expression. Pistachio intake prevents the impairment of lipid metabolism-related gene expression, in liver and adipose tissue. Representative images of the RT-PCR results (left panel) and mRNA levels of SREBP-1c, PPAR-ɣ, FAT-P, FAS and SCD-1 (right panel) in the livers (A-B) and adipose tissues (C-D) of lean, HFD and HFD-P mice (B). Data are the means ± S.E.M. (n = 8/group). * $p < 0.05$ *vs* lean; # $p < 0.05$ *vs* HFD.

Discussion

The present study demonstrates that pistachio consumption is able to prevent and to improve obesity-related metabolic dysfunctions such as dyslipidemia, hepatic steatosis and adipose tissue alterations in HFD obese mice. These beneficial effects could be due to the positive modulation of lipid metabolizing gene expression.

Compared to other nuts, pistachios represent a potentially functional food in preventing obesity-related metabolic dysfunctions. They are, in fact, a rich source of unsaturated fatty acids

and antioxidant substances, such as γ-tocopherol, β-carotene, lutein, selenium, flavonoids and phytosterols (Terzo et al., 2019; Tokuşoglu et al., 2005; Dreher 2012).

Indeed, *in vitro and in vivo* studies have highlighted pistachio consumption healthy properties, that could be attributed to the content of antioxidant substances (proanthocyanidins, epicatechin and isoquercetin, γ-tocopherol) (Alturfan et al., 2009; Gentile et al., 2012; Gentile et al., 2015; Paterniti et al., 2017, Zhang et al., 2010;). Moreover, several studies on humans provided evidence for beneficial effects of pistachio consumption on cardiovascular risk markers, including blood lipid levels (Aldemir et al., 2011; Gebauer et al., 2008; Sari et al., 2010), blood pressure (West et al., 2012), oxidative stress (Kocyigit et al., 2006) endothelial dysfunctions (Sari et al., 2010) and glucose dysmetabolism (Wang et al., 2012; Kendall et al., 2014).

Our goal was to evaluate pistachio intake ability of preventing (from obesity induction starting) metabolic obesity-related disorders in HFD mice.

In our experimental conditions, pistachio consumption did not modify the HFD-induced body weight increase as well as the food intake, in according to the epidemiological studies showing that regular intake of pistachios is not related to weight gain (Gulati et al., 2014; Kocyigit et al., 2006; Sari et al., 2010; Sheridan et al., 2007; Wang et al., 2012).

Moreover, pistachio intake failed to prevent the HFD-induced hyperglycaemia. Previous studies have reported contradictory results about the pistachio effects on glucose metabolism, depending on the subjects examined (healthy or affected by MetS) or on the temporal length of pistachio regular intake (Wang et al., 2012; Sari et al., 2010; Gulati et al., 2014). Indeed, only a long-term consumption appears to have beneficial effects on the obesity-related glucose dysmetabolism (Gulati et al., 2014), although in our experiments, 16 weeks of HFD-P did not improve the HFD-induced hyperglycaemia.

In the present study pistachio intake is able to prevent the plasma dyslipidemia and the lipid accumulation in liver and adipose tissue, providing evidence for lipid lowering properties of pistachios. These findings are in agreement with previous results obtained on animals (; Aksoy et al., 2007; Alturfan et al., 2009) and on humans (Aldemir et al., 2011; Edwards et al., 1999; Gebauer et al., 2008; Hernández-Alonso et al., 2015; Holligan et al., 2014; Kocyigit et al., 2006; Sari et al., 2010).

Different mechanisms could be responsible for the pistachio hypolipidemic effects. They could be related to the high content of MUFA and PUFA because it is well known that the consumption of unsaturated fatty acids reduces plasma LDL and triglyceride levels (Silva Figueiredo et al., 2017). Alternatively, or in addition, they could be due to the high levels of phytosterols. In fact, a diet-supplemented with phytosterols causes inhibition of

cholesterol absorption in the gastrointestinal tract (Altmann et al., 2004).

Although a high intake of nuts, in particular walnuts, can improve liver function in patients with hepatic steatosis and be positively linked to a lower risk of NAFLD developing (Gupta et al., 2015; Han et al., 2014), there are no data available about pistachio regular intake and hepatic function. Our results show for the first time that pistachio consumption exerts preventive and improving effects on the hepatic steatosis, on the fat liver accumulation and hepatic functions. In fact, liver index and ALT and AST plasma levels resulted significantly lower in pistachio-fed obese mice.

It is well accepted that the dietary fat composition influences the expression of gene controlling hepatic lipid metabolism (Jump, 2011; Nguyen et al., 2008). We investigated if the pistachio beneficial effects on hepatic steatosis could be due to changes in the expression of the transcription factors *PPAR-γ*, *SREBP-1c* with their target genes *FAS*, and *SCD1*, which are the principal regulators of fatty acid synthesis, and *FAT-P*, which is involved in the fatty acid uptake from the extracellular milieu (Jia et al., 2007).

Our RT-PCR analysis revealed that gene expression of *PPAR-γ*, *SREBP-1c*, *FAS*, *SCD1* and *FAT-P* was up-regulated in HFD liver compared to STD mice, confirming that HFD-induced impairment of the lipid metabolizing gene expression is involved in steatosis development (Lai et al., 2015; Lee et al., 2015; Oliveira Andrade et al., 2014; Xia et al., 2016; Zhuang et al., 2017). However, HFD-P was able to prevent *PPAR-γ*, *FAS* and *SCD1* up-regulation in obese liver suggesting that pistachio consumption exerts hypolipidemic effects by preventing hepatic *de novo* lipogenesis impairment, and to ameliorate severe steatosis by reducing FAT-P gene expression and consequently the fatty acid uptake.

Our results represent the first experimental data showing the pistachio ability of modulating lipid gene expression. On the other hand, several plant bioactive components (Hong et al., 2018a; Lee et al., 2015; Xia et al., 2016) as well as functional foods (Ayoub et al., 2018; Hong et al., 2018a;) able to counteract hepatic steatosis by positively modulating expression of genes linked to the lipid metabolism.

Moreover, our results suggest that pistachios-based diet is able to prevent fat mass accumulation because VAT, fat-mass, fat-index and adipocyte diameter were significantly reduced in HFD-P mice in comparison with HFD mice. Interestingly, in HFD-P mice SAT volume was increased, suggesting that pistachio consumption could be responsible of an adipose tissue redistribution linked to a healthier profile. In fact, only VAT increase has been reported to be strictly associated with cardiometabolic risk (Abraham et al., 2015; Bays, 2014). The role of functional foods or natural extracts on the specific regional adiposity had never been clearly

established. Just a multi-ethnic study revealed a link between a healthy dietary pattern (including nuts) and a lower visceral fat, although excluding subcutaneous fat reduction (Shah et al., 2016). Then our results are the first evidence regarding the ability of a functional food to influence adipose tissue redistribution.

Lastly our results provide evidence for a modulator role of regular pistachio intake on the expression of genes invoved in lipid metabolism also in adipose tissue. In fact, pistachio addition to the diet significantly prevented HFD-induced up-regulation of *SREBP-1c*, *P-PARγ* and *FAT-P* and reduced *FAS* and *SCD1* over-expression, suggesting decrease in de novo lipid synthesis and lipid uptake in the adipose tissue.

Conclusions

In conclusion, our results suggest that pistachio may act as an effective functional food, useful to prevent as well as to ameliorate obesity-related metabolic disorders such as hyperlipidemia, hepatic steatosis and adipose tissue fat accumulation. Regular pistachio intake could exert beneficial effects on the lipid metabolism by reducing the expression of lipid metabolism-related genes in liver and adipose tissue.

II° ARTICLE

PISTACHIO CONSUMPTION ALLEVIATES INFLAMMATION AND IMPROVES GUT MICROBIOTA COMPOSITION IN HIGH FAT DIET FED MICE

Disclosure

The results concerning this paper have been published in: *International Journal of Molecular Sciences* (Terzo et al., 2020). Therefore, in according to the journal style, the relative Methods will be described as last section of the article. Some preliminary results have been published in abstract form (Terzo et al., 2018 a).

Summary

The aim of the present study was to evaluate if the chronic intake of pistachio prevents the obesity-associated inflammation and the dysbiosis in HFD fed mice. Three groups of male mice (four weeks old; n=8 per group) were fed for 16 weeks with standard diet (STD), HFD or HFD supplemented with pistachios (HFD-P; 180 g/Kg of HFD). Serum, hepatic and adipose tissue inflammation markers were analyzed in HFD-P animals and compared to HFD and STD groups. Measures of inflammation, obesity, and intestinal integrity were assessed. Fecal samples were collected for gut microbiota analysis. Serum TNF-α and IL-1β levels were significantly reduced in HFD-P compared to HFD. Number and area of adipocytes, crown-like structure density, IL-1β, TNF-α, F4-80 and CCL-2 mRNA expression levels were significantly reduced in HFD-P subcutaneous and visceral adipose tissue compared to HFD. Significant reduction in the number of inflammatory foci and IL-1β and CCL-2 gene expression was observed in the liver of HFD-P mice compared with HFD. *Firmicutes/Bacteroidetes* ratio was reduced in HFD-P mice in comparison with HFD group. Pistachio diet significantly increased abundance of healthy genera such as *Parabacteroides, Dorea, Allobaculum, Turicibacter, Lactobacillus* and *Anaeroplasma*, while strongly reduced bacteria associated with inflammation such as *Oscillospira, Desulfovibrio, Coprobacillus* and *Bilophila*. The intestinal conductance was lower in HFD-P mice than HFD suggesting an improvement in the gut barrier function. The results of the present study show that regular pistachio consumption improves inflammation in obese mice. The effects could be related to positive modulation of the microbiota composition.

1. Introduction

The obesity and overweight in Western societies and developing countries have become one of the most important public health problems. They, in part, result from the consumption of unbalanced hypercaloric diets causing an excessive visceral fat accumulation (Hruby & Hu, 2015). Obesity is associated with chronic low-grade inflammation, which can impair glucose and fatty acid metabolism, leading to insulin resistance and metabolic syndrome (Eckel RH et al., 2005). Most studies have focused on adipocytes as the source of inflammatory mediators in this pathology. Storage of excess of triacylglycerol induces hyperplasia and hypertrophy of the adipocytes with altered release of adipokines and pro-inflammatory cytokines, that in turn enhance the recruitment of immune cells, especially macrophages (Trayhurn & Wood, 2004). Therefore, the macrophages in obese adipose tissue are considered the major source of pro-inflammatory cytokines, such as TNF-α and IL-6, which are involved in the abnormal metabolism (Lackey & Olefsky, 2015).

However, recent studies have suggested that changes in the composition of the gut microbiota are associated with the development of the metabolic disorders associated to obesity (Cani et al., 2012; Ridaura et al., 2013; Álvarez-Mercado et al., 2019). Indeed, a diet rich in saturated fat and poor of fiber is responsible of weight gain, changes in gut microbiota (Gentile & Weir, 2018) and increased intestinal permeability (Myles, 2014). The intestinal barrier dysfunction causes increased flow of lipopolysaccharides (LPS) deriving from gram-negative bacteria, towards the circulation (Moreira et al., 2012; Cani et al., 2007). In turns, LPS spread participates to metabolic endotoxemia development, adipose tissue dysfunction and systemic inflammation, triggering the obesity-related complications (Clemente-Postigo et al., 2019).

Nutritional strategies can represent a valid support to prevent metabolic and inflammatory diseases. Increased consumption of fruit and vegetables could prevent chronic diseases such as cardiovascular disease and prevent body weight gain (Tian et al., 2018). Additionally, plant-based foods reduce metabolic syndrome risk (Rizzo et al., 2011). Functional food, that is able to modulate the richness and the biodiversity of the gut microbiota, and consequently able to induce a healthier metabolic status, has received increased attention from researchers worldwide (Roopchand et al., 2015; Anhệ et al., 2015). It is widely accepted that the consuming nuts such as almonds, walnuts and pistachios, as a part of daily diet providing beneficial effects on human health (De Souza et al., 2017). Among nuts, Pistachio (*Pistacia vera* L.) results the healthiest, because of its fatty acid composition and bioactive compound content (such as lutein and anthocyanin) (Dreher, 2012; Terzo et al., 2019). In recent years, anti-inflammatory effects of the pistachios and anti-inflammatory activity of its components have been object of numerous studies. In particular, the anti-inflammatory effects have been reported both in *in vitro* models (Paterniti et al., 2017; Gentile et

al., 2007) and in various animal models (Ahmad et al., 2010; Esmat et al., 2012; Naouar et al., 2016). Antimicrobial properties of polyphenolic fractions obtained from roasted pistachios have been also demonstrated (Bisignano et al., 2013; Smeriglio et al., 2017).

Moreover, we have already shown that the daily pistachio intake prevents and improves some obesity-related metabolic dysfunctions such as dyslipidemia and hepatic steatosis in mice with induced-diet obesity, through a positive modulation of lipid metabolizing gene expression (Terzo et al., 2018). Nevertheless, no study has characterized the links between pistachio supplementation, adiposity-related inflammation and gut microbiota alterations. High fat diet (HFD) mice are considered a good obese model to characterize the beneficial potential of various treatment on obesity-related disorders because they develop dyslipidemia, hyperglycemia (Baldassano et al., 2013; Baldassano et al., 2015), type 2 diabetes mellitus (Rossmeis et al., 2003), hepatic steatosis (Amato et al., 2017), atherosclerosis (Oppi et al., 2019), and neurodegeneration (Nuzzo et al., 2015).

Therefore, the purpose of the present study was to investigate whether chronic pistachio consumption is able to prevent the associated-visceral-obesity inflammation, the altered composition of gut microbiota and the intestinal barrier integrity in HFD-obese mice.

2. Results

2.1 Impact of pistachio consumption on body weight and metabolic parameters

As previously reported (Terzo et al., 2018; Amato et al., 2017), after 16 weeks on HFD, mice showed a significant increase in body weight, triglyceride and cholesterol plasma concentration in comparison with standard diet (STD)-fed lean animals. In HFD supplemented with pistachio (HFD-P)-fed mice, triglyceride and cholesterol concentrations were significantly reduced in comparison with untreated obese mice, whereas body weight and food intake were similar (Table 1).

Table 1. Effects of Pistachio consumption on HFD-related dysmetabolisms.

	Lean	**HFD**	**HFD-P**
Body weight (g)	32.3 ± 0.9 g	46.2 ± 1.1 g *	46 ± 1.2 g *
Food Intake (g)	4.05 ± 0.2 g	3.4 ± 0.08 g	3.3 ± 0.07 g
Triglycerides (mg/dl)	82 ± 4.5 mg/dl	119 ± 5.5 mg/dl *	93.1 ± 5.1 mg/dl #
Cholesterol (mg/dl)	100 ± 5 mg/dl	192 ± 4 mg/dl *	150 ± 4 mg/dl #

Body weight, Food Intake, triglyceride and cholesterol plasma concentrations of Lean, HFD and HFD-P animals at the end of the experimental period. Data are expressed as mean ± SEM (n = 8 / group). * P <0.05 compared with lean; # P <0.05 compared with HFD.

2.2 Impact of pistachio consumption on TNF-α and IL-1β expression

To examine whether pistachio consumption prevents the systemic inflammation induced by HFD, serum levels of the pro-inflammatory cytokines TNF-α and IL-1β were evaluated by ELISA. As shown in Figure 1, intake of pistachios significantly decreased the HFD-induced high levels of TNF-α and IL-1β.

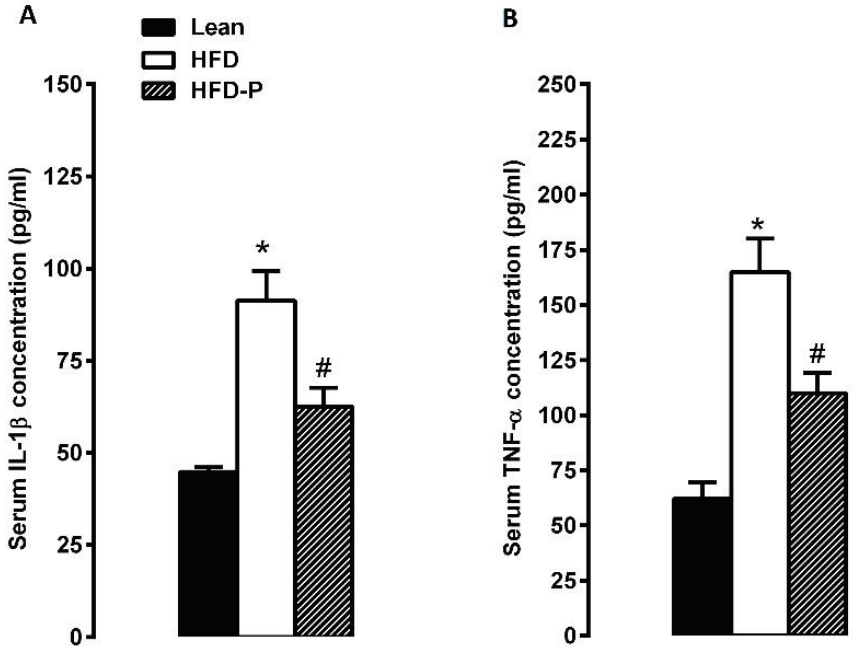

Figure 1. Effects of pistachio consumption on pro-inflammatory cytokines. Serum circulating levels of IL-1β (**A**) and TNF-α (**B**) in Lean, HFD and HFD-P. Data are expressed as mean ± SEM; (n = 8/group). * P <0.05 compared with lean; # P <0.05 compared with HFD.

2.3 Impact of pistachio consumption on adipocytes hypertrophy

Adipocyte area (μm^2) and adipocyte size distribution (%) were analyzed in visceral (VAT) and subcutaneous (SAT) adipose tissues. The adipocytes area in the HFD was significantly higher than in the lean group; however, the degree of increase was significantly suppressed by HFD-P suggesting that pistachio chronic intake reduces the hypertrophy in both fat depots examined (Figure 2A, 2B, 2C).

2.4 Impact of pistachio consumption on adipose and hepatic tissue inflammation

The presence of CLS, as index of macrophage infiltration, was evaluated and quantified in VAT and SAT. As shown in Figure 3, more crown-like structures were detected in HFD mice,

compared to lean animals. Interestingly, in HFD-P mice the CLS density was significantly lower in comparison with HFD adipose tissues (Figure 3A, 3B). Furthermore, RT-PCR analysis revealed significantly higher levels of IL-1β, TNF-α, F4-80 and CCL2 mRNA in HFD mouse VAT and SAT than lean mice. However, pistachio-diet reduced the increase of the pro-inflammatory cytokines and the macrophage infiltration markers in both adipose tissue depots (Figure 3C).

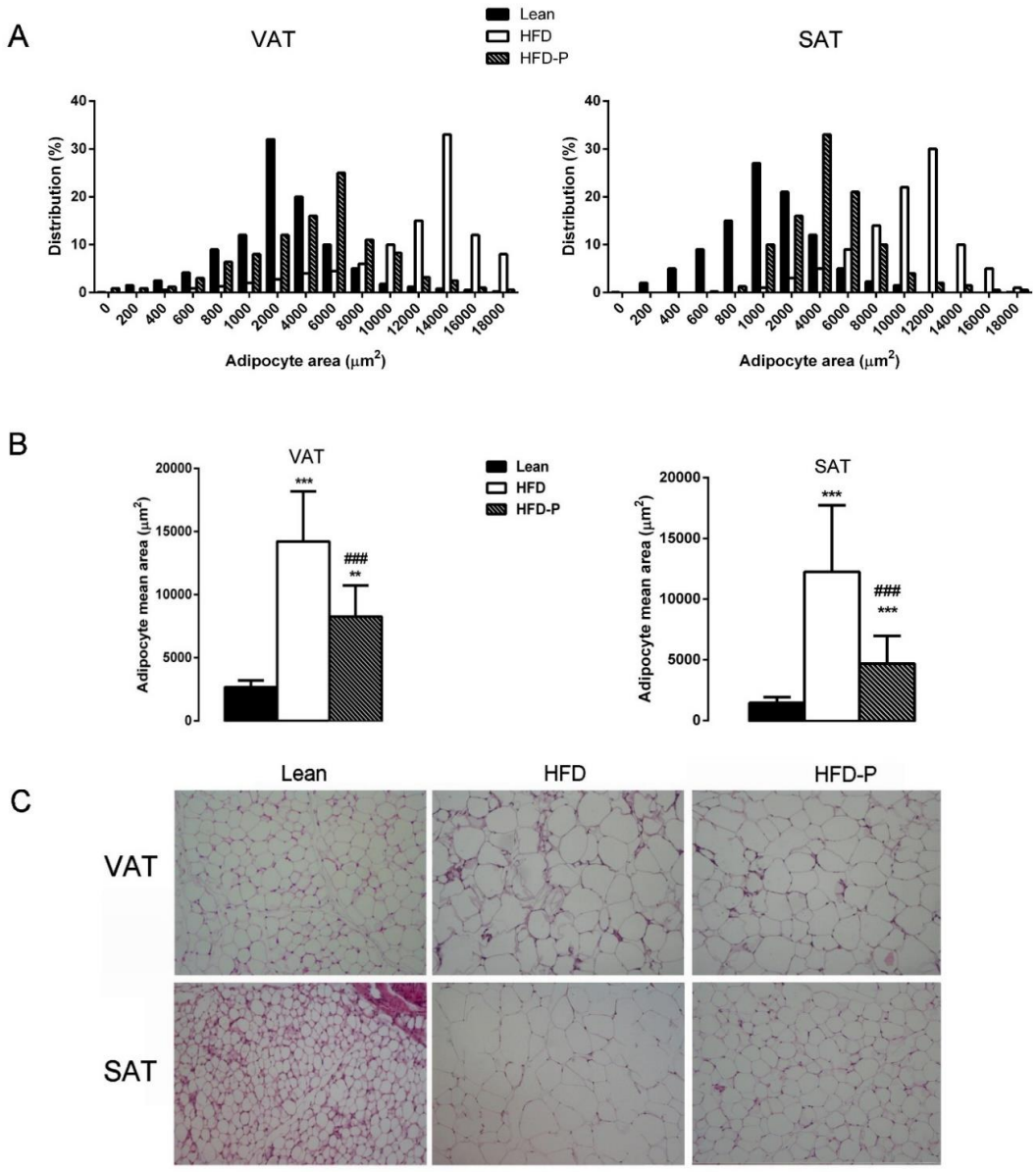

Figure 2. Effects of pistachio consumption on adipocyte morphology. (**A**) Adipocyte size distribution (%) and (**B**) adipocyte mean area (μm^2) of epididimal (VAT) and subcutaneous adipose tissue (SAT) in Lean, HFD and HFD-P. (**C**) Adipose tissue staining (H&E staining, magnification 10X) in Lean, HFD and HFD-P. Data are expressed as mean ± SEM; (n = 8/group). * P <0.05 compared with lean (* p < 0.05; ** p < 0.01; *** p < 0.001); # P <0.05 compared with HFD (# p < 0.05; ## p < 0.01; ### p< 0.001).

As previously reported (Terzo et al., 2018), pistachio consumption counteracted the hepatic steatosis development consequent to HFD (Figure 4A). HFD mice showed higher infiltration of inflammatory cells in the liver compared to STD animals. Anyway, infiltration was reduced in HFD-P livers in comparison with HFD (Figure 4A, 4B). Moreover, pistachio intake significantly prevented the increase in hepatic mRNA levels of IL-1β and CCL2 observed in the HFD liver comparatively to STD animals (Figure 4C).

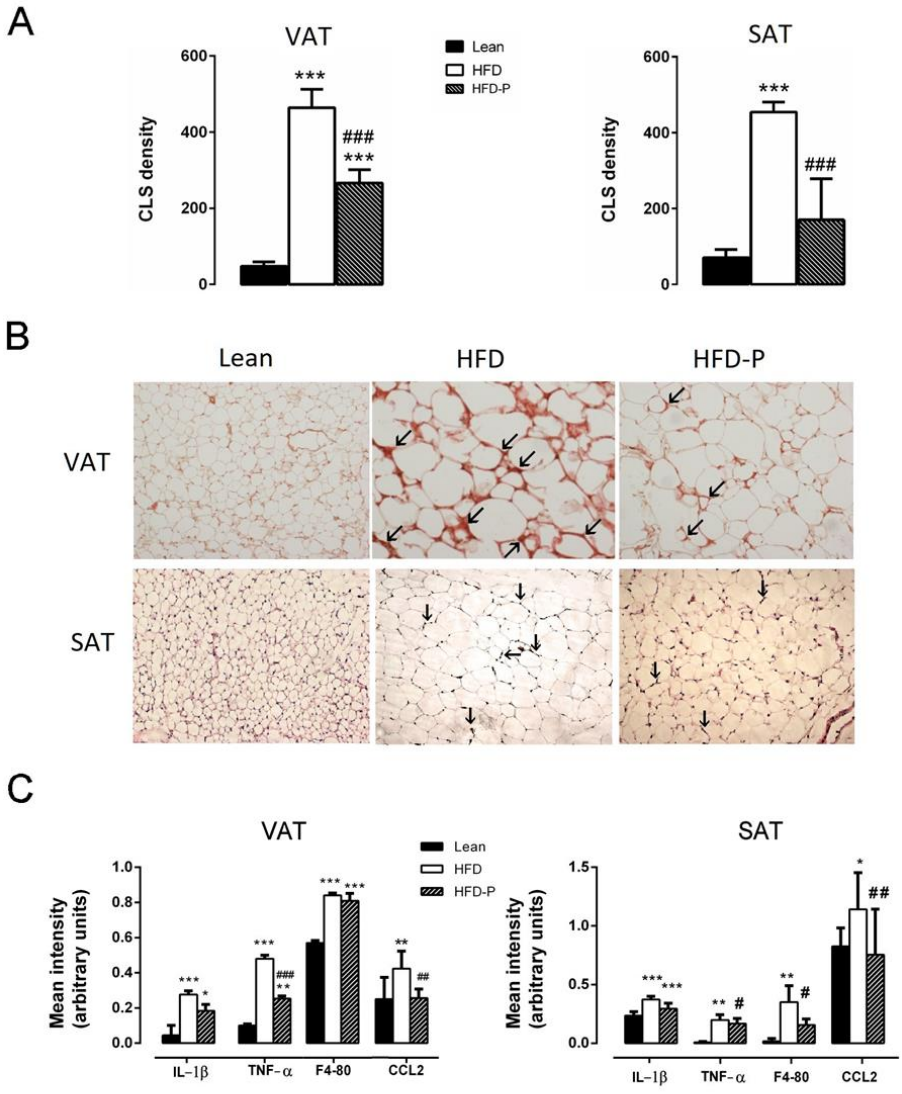

Figure 3. Effects of pistachio consumption on CLS density. (**A**) Representative results of the density of MAC-2 positive CLS stained in epididimal (VAT) and subcutaneous (SAT) adipose deposits of the three groups of animals (CLS number/10.000 adipocytes). (**B**) VAT and SAT IHC analysis for MAC-2 positive macrophages forming CLS (arrows) in Lean, HFD and HFD-P animals (magnification 10X). (**C**) Effect of Pistachio consumption on IL-1β, TNF-α, F4-80, and CCL2 mRNA expression in VAT and SAT of Lean, HFD, and HFD-P mice. Data are expressed as mean ± SEM; (n = 8/group). * P <0.05 compared with lean (*p < 0.05; ** p < 0.01; *** p < 0.001); # P <0.05 compared with HFD (# p < 0.05; ## p < 0.01; ### p< 0.001).

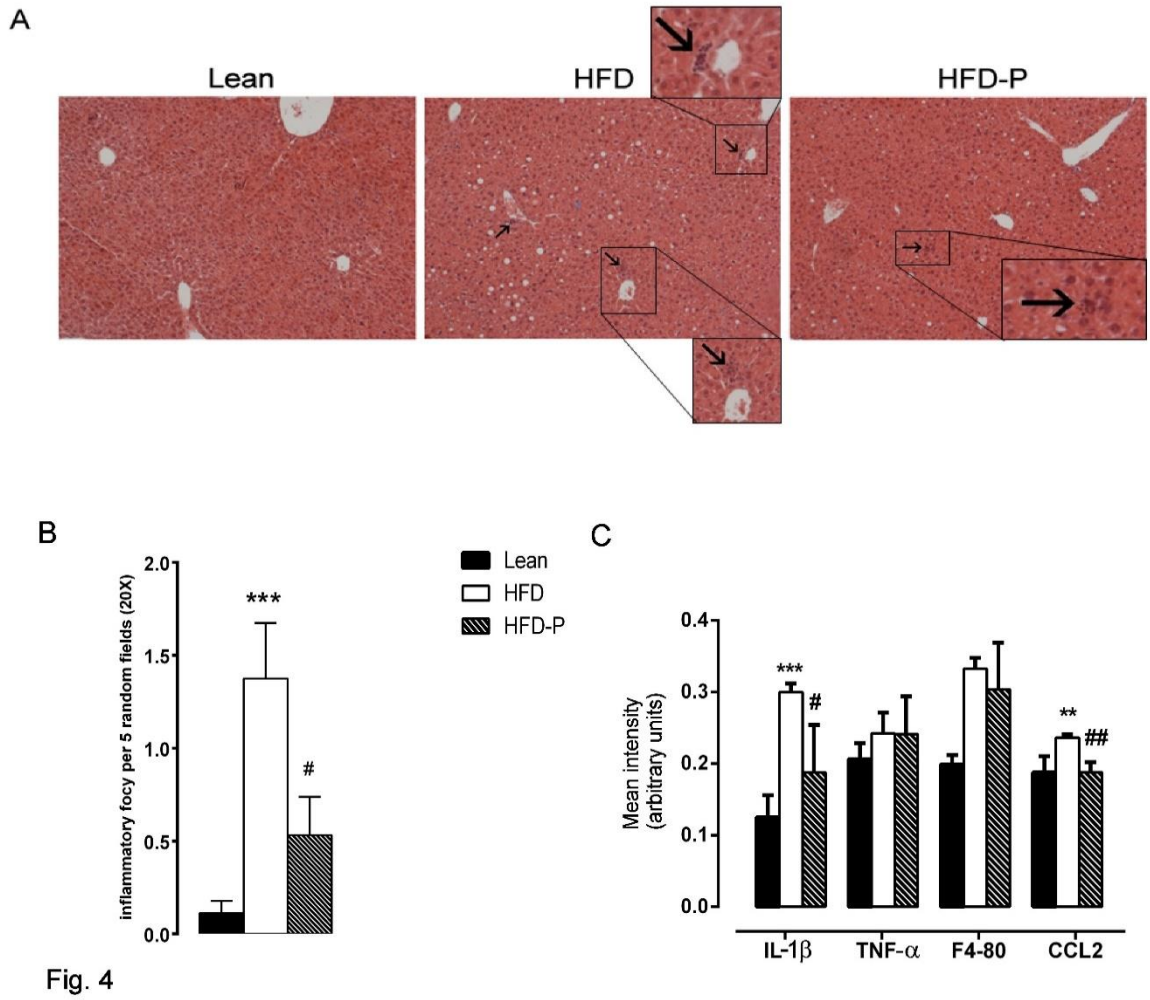

Fig. 4

Figure 4. Effect of pistachio consumption on liver inflammation. (**A**) Liver histology of Lean, HFD, and HFD-P mice was examined by H&E staining. Arrows indicate points to inflammatory foci (magnification 10X). (**B**) Quantification of inflammatory foci per 5 random fields under 20X magnification. (**C**) mRNA levels of IL-1β, TNF-α , F4-80, and CCL2 in the livers of Lean, HFD, and HFD-P mice (B). Data are the means ± SEM. (n = 8/group). * P <0.05 compared with lean (*p < 0.05; ** p < 0.01; *** p < 0.001); # P <0.05 compared with HFD (# p < 0.05; ## p < 0.01; ### p< 0.001).

2.5. Impact of pistachio consumption on gut microbial community

To examine the changes of the gut microbiota in response to pistachio diet in obese HFD mice, we analyzed microbial composition in the feces of mice fed STD- HFD- and HFD-P, by NGS analysis. After 16 weeks of HFD feeding, a decrease in the phyla *Bacteroidetes* and an increase in the phyla *Firmicutes* and *Proteobacteria* relative to STD were observed both in HFD group and in HFD-P mice (Figure 5A). The ratio of *Firmicutes* to *Bacteroidetes* was significantly higher in HFD group than lean mice, consistent to the microbial changes of the two phyla in mice with HFD-induced obesity. Although this value was index of dysbiosis also in HFD-P group, it was significantly improved by pistachio intake (Figure 5B). Interestingly, *Tenericutes* abundance of HFD-P mice was significantly increased in comparison with the HFD control mice; on the contrary pistachio diet significantly reduced *Proteobacteria* abundance (Figure 5A).

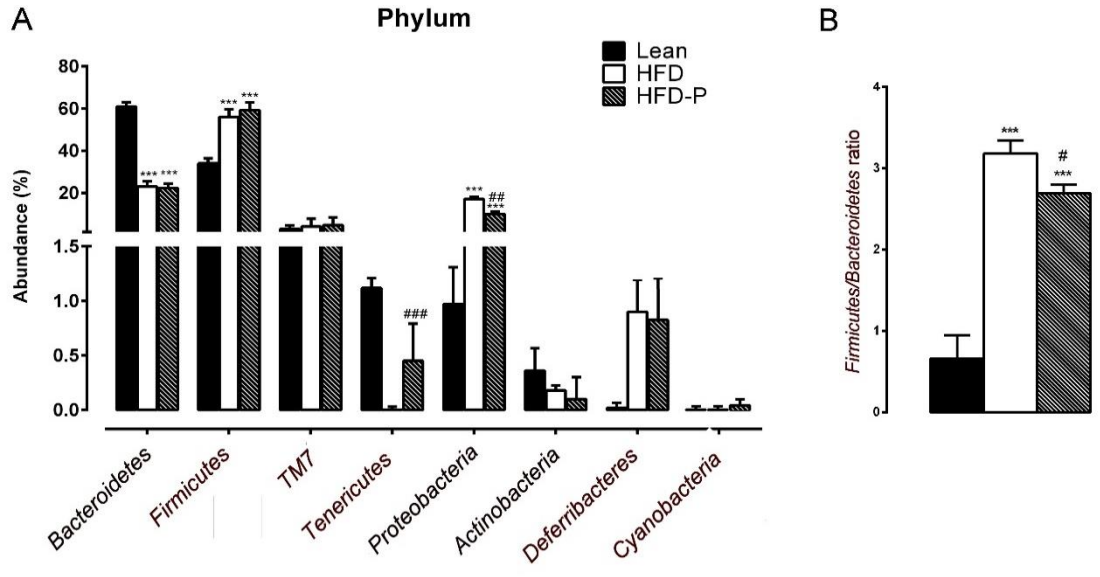

Figure 5. 16S rDNA sequencing of bacterial DNA in the feces of Lean, HFD and HFD-P to discriminate the intestinal microbial profile. (**A**) Graphic representation of the relative abundance (%) of gut microbiota phyla composition of the three groups of animals. (**B**) Ratio of Firmicutes to Bacteroidetes in Lean, HFD and HFD-P. Data are expressed as mean ± SEM; (n = 8 / group). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$); hash denotes significant difference compared with the HFD group (# $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$).

At the genus level, pistachio diet altered in a significantly positive manner the abundances of 10 genera, compared with the HFD animals. In particular abundance of *Parabacteroides*, *Dorea*, *Allobaculum*, *Turicibacter*, *Lactobacillus* and *Anaeroplasma* genera was enhanced, while *Oscillospira, Desulfovibrio, Coprobacillus* and *Bilophila* abundance was reduced in comparison with HFD mice (Figure 6).

2.6 Impact of pistachio consumptiom on intestinal barrier

Barrier integrity in small intestine sections was evaluated in an Ussing chamber system by conductance measurements of mucosal preparations from all experimental groups. Conductance values in duodenal sections from animals fed HFD were significantly higher than those from the STD group (about 60% increase). Notably, conductance values from the HFD-P group were significantly lower than HFD group and very similar to lean group (Figure 7).

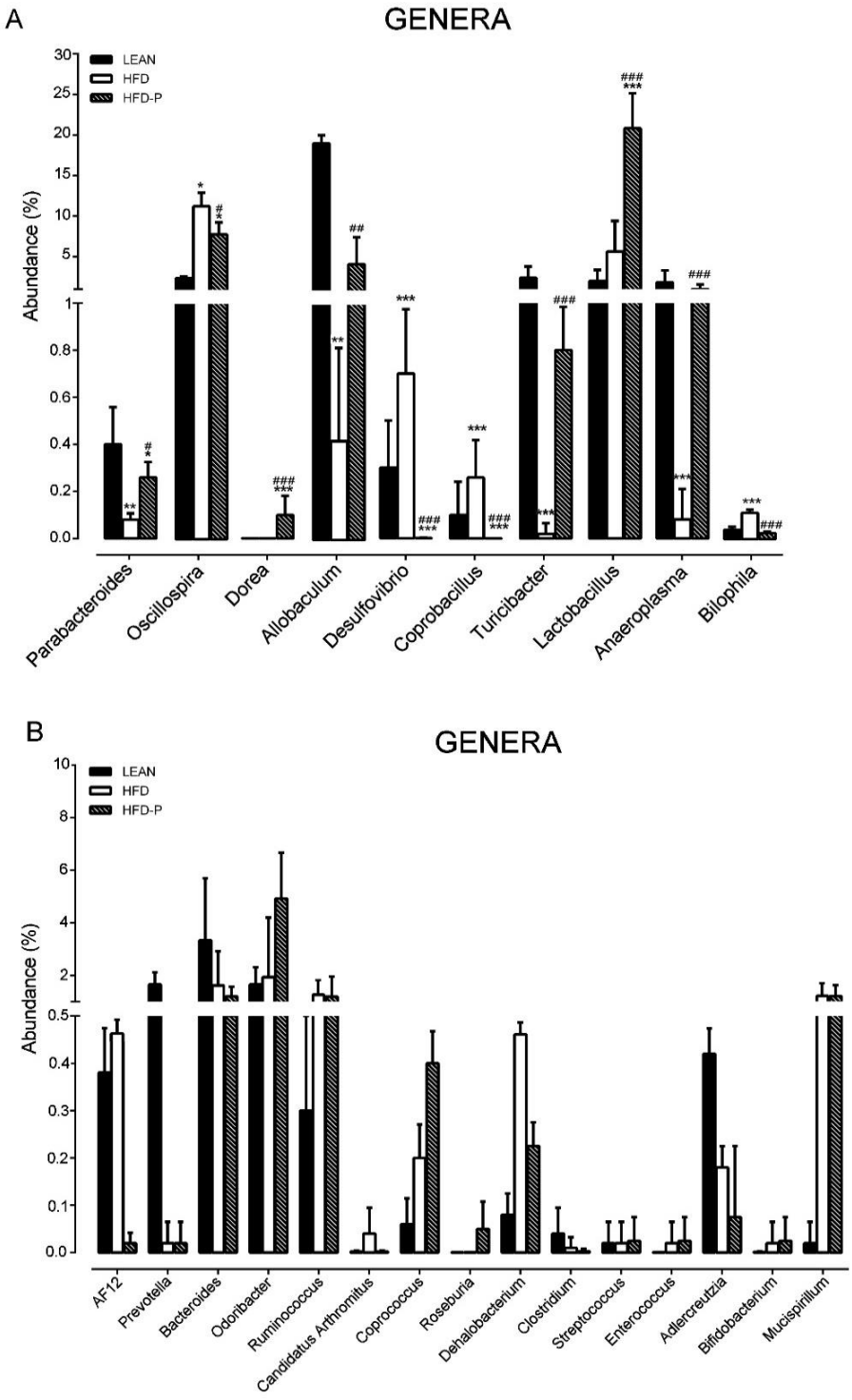

Figure 6. Genus level taxonomic distributions of the microbial communities in the feces of Lean, HFD and HFD-P. (**A**) Genera abundance (%) which was significantly modified by pistachio intake. (**B**) Genera abundance (%) which was not modified by pistachio intake. Data are expressed as mean ± SEM; (n = 8 / group). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$); hash denotes significant difference compared with the HFD group (# $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$).

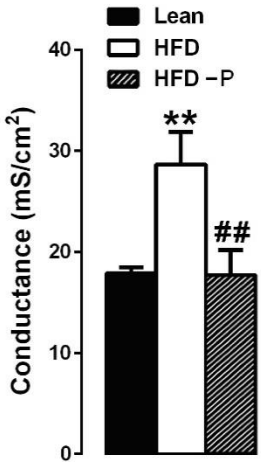

Figure 7. Effect of pistachio consumption on the conductance of isolated duodenal sections from Lean, HFD and HFD-P mice by the Ussing chambers technique. Data are expressed as mean ± SEM; (n = 8 / group). (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$); hash denotes significant difference compared with the HFD group (# $p < 0.05$; ## $p < 0.01$; ### $p < 0.001$).

3. Discussion

The present study provided evidence that regular pistachio intake in HFD-fed obese mice ameliorates systemic and metabolic tissue inflammation, it positively modulates the gut microbial composition, and it increases the intestinal barrier function.

Previous *in vitro* and *in vivo* studies have examined the antioxidant, anti-inflammatory and anti-apoptotic potential of pistachio (Gentile et al., 2007; Gentile et al., 2015; Hong et al., 2018b; Mehla et al., 2011; Yayeh et al., 2012; Zhang et al., 2010). In particular, the pistachio properties were tested on carrageenan or LPS-induced acute inflammatory response (Ben Khedir et al., 2016; Gentile et al., 2012), inflammatory bowel disease and colitis (Kim & Neophytou, 2009; Naouar et al., 2016; Papalois et al., 2012; Tafti et al., 2017), cancer (Balan et al., 2007; Catalani et al., 2017; Spyridopoulou et al., 2017), allergic inflammation in asthmatic model (Qiao et al., 2011). To our knowledge, the present study is the first one exploring the anti-inflammatory effects of pistachios in mice with HFD-induced obesity.

Obesity is characterized by chronic low-degree inflammation. In fact, excessive calorie intake increases fat accumulation and the lipotoxicity activates the production of cytokines and cells involved in innate immunity. This production promotes a chronic, low-grade inflammatory status, induces recruitment and activation of mature immune cells and other cells, such as macrophages and adipocytes respectively, that modify the tissue and reinforce the inflammatory response (Clemente-Postigo et al., 2019, Lumeng & Saltiel, 2011).

We previously reported that a pistachios-based diet exerts beneficial effects in HFD obese mice. In fact, it reduces the dyslipidemia, hepatic steatosis and is able to prevent and/or to improve visceral fat mass accumulation in HFD mice, through a redistribution towards the subcutaneous fat

depot, which is indicative of a healthier profile (Terzo et al., 2018). The present work not only confirms that the pistachio diet modifies fat depots, as suggested by the morphological analysis of visceral and subcutaneous adipose tissue, but also reduces the obesity-linked inflammatory status.

First, we highlighted that pistachio diet significantly prevents the increase of pro-inflammatory cytokines, TNF-α and IL-1β, induced by HFD in the systemic circulation. Furthermore, we provided evidence that visceral and subcutaneous adipose tissue and liver inflammation induced by obesity was strongly prevented by pistachio intake. Various inflammatory mediators are involved in adipose tissue and liver inflammation. In the adipose tissue, a paracrine loop linking fatty acids, TNF-α and CCL2 establishes a vicious cycle between adipocytes and macrophages that aggravates inflammation (Balan et al., 2007). In the liver, the increased influx of fatty acids induces lipotoxic injury and activation of inflammatory response. Accordingly, abundant expression of proinflammatory cytokines IL-1β and TNF-α is often associated with NAFLD (Haynes et al., 2004).

We found that HFD-P mice exhibit lower levels of TNF-α, F4-80 and CCL2 as well as minor macrophage infiltration, detected as CLS density, in the adipose tissue in comparison to HFD animals. Also in the liver, we found a reduction of IL-1β and CCL2 mRNA levels and a decreased number of inflammatory foci in comparison with HFD mice. These changes would favor an anti-inflammatory microenvironment able to counteract the biochemical dysfunctions occurring in adipose tissue or in the liver of HFD mice.

Obesity and metabolic disorders are complex processes, involving also the crosstalk between gut microbiota and host metabolism (Marchesi et al., 2016). The gut microbiota may induce inflammation in visceral adipose tissue via LPS and TLR4 signaling pathways with increased macrophage infiltration and release of a variety of pro-inflammatory mediators, which in turn recruit additional macrophages to further propagate the chronic inflammatory status (Olefsky & Glass, 2010; Caesar et al., 2015). Therefore, in attempt to elucidate an eventual contribution of the gut microbiota to the beneficial pistachio effects, we investigated the profiling changes of the gut microbiota composition in mice, performing 16S rDNA sequencing by NGS analyses. Indeed, pistachio consumption is able of modifying human gut microbiota composition by increasing the number of potentially beneficial butyrate-producing bacteria (Ukhanova et al., 2014). Our results demonstrate that the microbial communities were influenced by different type of diets. Analysis at the phylum level indicated that the fecal microbiota was dominated by seven major phyla: *Firmicutes, Bacteroidetes, Proteobacteria, TM7, Deferribacteres, Actinobacteria* and *Tenericutes*. We observed a dramatic reduction in abundance of *Bacteroidetes* and a marked increase in *Firmicutes* in HFD group, in according to the increased *Firmicutes* to *Bacteroidetes* ratio identified

in obese humans and mice (Ley et al., 2005; Delzenne et al., 2010). However, although the pistachio diet failed to maintain the *Firmicutes/Bacteroidetes* proportion observed in STD mice, the ratio value in HFD-P was significantly lower than HFD group suggesting a pistachio protective effect against dysbiosis.

Interestingly, compared with the HFD control mice, we found a significant increase in the abundance of *Tenericutes* and a significant decrease in the abundance of *Proteobacteria* phylum in HFD-P mice. Bacteria from the *Tenericutes* phylum have been associated positively with the modulation of the mmune system induced by high-polyphenol content food, such as cocoa (Camps-Bossacoma et al., 2017) and lower counts of these bacteria were found in intestinal inflammation induced by dextran sodium sulphate (Nagalingam et al., 2011). Therefore, more relative abundance of *Tenericutes* induced by pistachio diet could provide some beneficial effects in the intestinal integrity. In addition, several reports have endorsed the abundance of *Proteobacteria* in the gut microbiota as a potential marker for obesity-related metabolic disorders in both humans and rodents (De Filippo et al., 2010; Ridaura et al., 2013). Therefore, the lower level of *Proteobacteria* in HFD-P-fed mice than HFD, could be indicative of less severe health condition.

At the genus level *Lactobacillus* was significantly increased in HFD-P in comparison with the other groups. The relative abundance of *Lactobacillus* caused by pistachio intake can be interpreted as a positive effect because *Lactobacillus* is a well-known probiotic that has been associated to reduced colitis in several model of inflammatory bowel diseases (Zhu et al., 2013) as well as to protective effect in the intestinal barrier function and steatosis (Guarner et al., 2005; Ritze et al., 2014; Zhang et al., 2012).

Interestingly, pistachio intake improved the abundance of other genera usually associated to a positive impact on health host such as *Parabacteroides*, *Dorea, Allobaculum, Turicibacter* and *Anaeroplasma*. *Parabacteroides* is a genus predominantly found in the gut of healthy individuals and negatively correlated with body weight gain, liver steatosis and epididymal fat accumulation (Carbajo-Pescador et al., 2019). *Allobaculum* genus has been associated with a better mucus layer in the colon (Jakobsson et al., 2015), suggesting that its decrease reflects the alteration of the mucus layer in HFD. Then, pistachio diet might prevent this alteration. Moreover, *Allobaculum,* as well as *Dorea*, are among the major producer of butyrate, an important fuel for epithelial colonocytes and has been shown to help maintain normal differentiation. Thus, an increase in the amounts of butyrate generated in the gut might be an indication of improved health. Accordingly, butyrate-producing probiotics reduce NAFLD progression in rats (Endo et al., 2013) and attenuate HFD-induced steatohepatitis in mice by improving intestinal permeability (Hamer HM et al., 2008; Xu J et al., 2007; Yang C et al., 2019; Zhou D et al., 2018).

The decreased abundance of *Turicibacter* in HFD mice, which was prevented by pistachio intake, well fits with previous data showing a depletion of *Turicibacter* in animal models of inflammatory bowel disease and confirming the hypothesis that *Turicibacter* is an anti-inflammatory taxon (Jiao et al., 2018; Johnson et al., 2015; Liu et al., 2016). Recent data reports that *Anaeroplasma* abundance is decreased significantly in obese rat, while the increased abundance is related to a reduction in fat accumulation and inflammatory factors expression in the liver (Liang et al., 2018).

Another interesting effect of pistachio intake on gut microbiota concerns the decrease of genera associated with inflammation such as *Desulfovibrio, Coprobacillus, Oscillosphira,* and *Bilophila*. *Desulfovibrio* is a genus responsible of the 60% of the total hydrogen sulfide (H_2S) production in the colon. H_2S inhibits the mitochondrial respiration of colonic epithelial cells (Beaumont et al., 2016) reducing the diffusion of oxygen and then subtracting energy useful to the beta-oxidation of butyrate (Byndloss et al., 2017). Thus, it is likely to hypothesize that the reduction of H2S-producing bacteria by pistachio enhances the output of SCFAs, such as butyrate, improving intestinal health and inflammation (Guo et al., 2018). *Coprobacillus* has been reported to be correlated negatively with most of the features of obesity in obese rats (Li et al., 2019; Wang et al., 2018). Abundance of *Oscillospira* has been associated with systemic inflammation and altered intestinal permeability (Santisteban et al., 2017; Thevaranjan et al., 2017;) and diets rich of polyphenols improve HFD-induced liver steatosis by reducing *Oscillospira* abundance (Wu et al., 2018; David et al., 2014). *Bilophila* abundance seems to be related with colon inflammation (David et al., 2014). A recent work reports that the treatment with a phenolic compound alleviates obesity-related inflammation in HFD-mice, by inhibiting the expansion of bacteria *Bilophila* genus (Guo et al., 2018).

The changes in microbiota composition may be due to the different components of the pistachios such as fatty acids, flavonoids or fiber. Pistachios might exhibit prebiotic effects by enriching potentially beneficial microbes such as lactic acid bacteria.

Therefore, taken together these results suggest that the gut microbial alterations observed in HFD-P mice may be associated with pistachio metabolic and anti-inflammatory benefits.

It is interesting to note that an increased intestinal conductance was observed in small intestine of HFD mice in comparison with lean or HFD-P mice, suggesting that HFD induces a decrease in the intestinal epithelial integrity and an increased ability of ions and small molecules to permeate through the paracellular pathway. According to our data, several studies report an increased gut permeability in HFD mice (Devkota et al., 2012; Cani et al., 2009). The intestinal conductance value in HFD-P group was similar to lean group suggesting the pistachio diet ability of

preventing the increase in permeability and thus of exerting protective action of pistachio diet on the intestinal barrier function. Cani and collaborators (Cani et al., 2008) provided evidence that the development of metabolic endotoxemia and the linked metabolic disorders induced by high-fat feeding are associated with an increased intestinal permeability. Therefore, it is likely to hypothesize that the modulation of gut bacteria associated with increased intestinal barrier function are involved in the anti-inflammatory effects of pistachio diet.

In conclusion, chronic intake of pistachio exerts beneficial effects in obese mice by alleviating inflammation in adipose tissues and liver and by impacting on the gut microbioma composition. In particular, it enhances the abundance of beneficial bacteria genera such as *Lactobacillus, Dorea, Allobaculum* and inhibited the growth of bacterial associated with obesity-related comorbidities and inflammation such as *Desulfovibrio* and *Bilophila*.

4. Material and methods

4.1. Animals and Diets

The procedures were performed in accordance with the conventional guidelines for animal experimentation (Italian D.L. No. 26/2014 and subsequent variations) and the recommendations of the European Economic Community (2010/63/UE). The experimental protocols were approved by the animal welfare committee of the Istituto Zooprofilattico Sperimentale della Sicilia “A. Mirri” (Palermo, Italy) and authorized by the Ministry of Health (Rome, Italy; Authorization Number 349/2016-PR).

Four-week old male C57BL/6J (B6) mice, purchased from Harlan Laboratories (San Pietro al Natisone Udine, Italy) were housed in a room with controlled temperature and dark-light cycles, with free access to water and food. After acclimatization (1 week), the animals were weighed and divided into three groups: (1) Lean group: control animals fed standard diet (STD; 4RF25 Mucedola, Milan, Italy) for 16 weeks; (2) High-fat diet (HFD) group: obese animals fed HFD (PF4215, Mucedola, Milan, Italy) for 16 weeks. (3) HFD-P group: obese animals fed HFD supplemented with pistachio for 16 weeks. HFD-P was custom designed and prepared by Mucedola S.r.l (PF4215/C; R&S 34/16). It was obtained by substituting 20% of the caloric intake from HFD with pistachio (180 g/Kg of HFD). The HFD and HFD-P were stored in vacuum containers at 4°C. The energy densities of diets is shown in Table 2.

Table 2. Composition and energy densities of STD, HFD and HFD-P

Ingredient (g/kg)	STD	HFD	HFD-P
Total Energy, Kcal/g	3.5	6	6
Protein, %	20	20	20
Carbohydrate, %	70	20	20
Fat, %	10	60	60

STD, Standard diet. HFD, high fat diet. HFD-P, HFD supplemented with pistachio.

Pistachio nuts belong to *Pistacia vera* L. species and were purchased by Pistachio Valle del Platani Association and Pistacchio di Raffadali (Agrigento-AG, Sicily). As previously described (Amato et al., 2017), during the 16th weeks of the experiment, changes in body weight and food-intake were weekly measured and compared among the different groups of animals. At the end, the animals were sacrificed by cervical dislocation; the blood was collected immediately by intracardiac puncture, and plasma was isolated by centrifugation at 3000 rpm at 4 °C for 15 min and stored at -80 °C until analysis. Liver, adipose tissue and small intestine were rapidly removed; a part of each tissue was fixed in 4% neutral formalin solution for histological analysis and another part was stored at -80 °C for biomolecular analysis. Five-centimeter segments of small intestine were taken for Ussing chamber assays.

4.2. Plasma biomarker analysis

IL-1β and TNF-α were quantified by a commercial ELISA Kit (Cloud-Clone Corp, Wuhan, Hubei), based on the manufacturer's instructions. The levels of triglyceride and total cholesterol in serum were evaluated by using automatic biochemical analyser (ILab 600, Instrumentation Laboratory, Milano-Italia).

4.3. Liver and adipose tissues histology and immunohistochemistry

Hepatic, visceral (epididymal) and subcutaneous white adipose tissues (WAT) were fixed with 4% formaldehyde solution for 24 h and embedded in paraffin. Then, 5 μm sections were prepared and stained with haematoxylin and eosin (H&E) for morphological examination. The number of liver inflammatory foci was calculated by counting inflammatory cell aggregates in the hepatic lobules per 5 random fields at a magnification of 20X. Hepatic inflammatory foci are defined as aggregates of inflammatory cells that accumulate in the liver during chronic inflammation (Kleiner & Brunt, 2012; Wu & Siow, 2009). The number of adipocytes per microscopical field (density) was determined at a magnification of 20X. The mean surface area of the adipocytes (μm^2) was calculated using image analyser software (Visilog 6, Courtaboeuf,

France). Each adipocyte was manually delineated, and 700-1000 adipocytes per condition were assessed.

Images of H&E liver and WAT sections were captured using an optical microscope (Leica DMLB, Meyer instruments, Houston, Texas) equipped with a DS-Fi1 camera (Nikon, Florence, Italy) and were analysed at 10X and 20X magnification.

For immunohistochemistry, deparaffinised sections were treated with 3% hydrogen peroxide to inactivate endogenous peroxidase followed by rinse in PBS for 5 minutes. Subsequently, the sections were incubated with the primary antibody Mac-2 at 4 °C overnight (1:2800, Cedarlane, Ontario, Canada CL8942AP). After PBS washing, sections were incubated with the secondary antibody biotinylated (Anti-Mouse IgG/Rabbit IgG) (1:400, Vector Laboratories, BA-4001) for 30 minutes. Histochemical reactions were performed using Vector's Vectastain ABC Kit (Vector Labortatories, Burlingame, USA) and diaminobenzidine as substrate (Sigma, Milano, Italia). Crown-like structures (CLS) were counted as a measure of adipose tissue inflammation and expressed as number of CLS/10.000 adipocytes.

4.4. Reverse Transcription Polymerase Chain Reaction (RT-PCR)

RNA was extracted from liver, epididymal and subcutaneous adipose tissue using the RNeasy plus Mini Kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. The extraction from adipose tissues was performed after a preliminary step of lysis using Triazol. Two nanograms of total RNA were used for cDNA synthesis with High Capacity cDNA Reverse Transcription (Applied Biosystems, MA, USA). The target cDNA was amplified using genetic-specific primers, as listed in Table 3. The amplification cycles included denaturation at 95 °C for 45 s, annealing at 52 °C for 45 s, and elongation at 72 °C for 45 s. After 40 cycles, the PCR products were separated by electrophoresis on a 1.8% agarose gel for 45 min at 85 V. The gels were stained with 1 mg/mL ethidium bromide and visualized with ultraviolet (UV) light using E-Gel GelCapture (Thermo Fisher Scientific, Monza, Italy), and the expression levels of the gene targets, normalized to the endogenous reference (β-actin), were analysed using E-Gel GelQuant Express Analysis Software (Thermo Fisher Scientific, Monza, Italy).

Table 3. Oligonucleotide sequence of primers for RT-PCR.

Gene	Forward primer	Reverse primer	Size (bp)
IL-1β	5′-CAGGATGAGGACATGAGCACC-3′	5′-CTCTGCAGACTCAAACTCCAC-3′	450
TNF-α	5′-AGCCCACGTCGTAGCAAACCA-3′	5′-GCAGGGGCTCTTGACGGCAG-3′	260
F4-80	5′-GCCACGGGGCTATGGGATGC-3′	5′-TCCCGTACCTGACGGTTGAGCA-3′	360
CCL2	5′-TCTGTGCTGACCCCAAGAAGG-3′	5′-TGGTTGTGGAAAAGGTAGTGGAT-3′	183
β-actin	5′-GGATCCCCGCCCTAGGCACCAGGGT-3′	5′-GGAATTCGGCTGGGGTGTTGAAGGTCTCAAA-3′	289

4.5. Gut microbiota composition

Six hours before the sacrifice, the mice were keep individually in clean cage without food and stool samples were collected from each mouse for gut microbiota analysis using an autoclaved tube. Bacteria DNA was extracted from stool samples (200 mg per mouse) using the QIAamp DNA Stool Handbook kit (QIAGEN, Milan-Italy) following the manufacturer's protocol. The extracted DNA was used for the metagenomic study carried out by the BMR Genomics company s.r.l. (Padova-Italy).

For NGS sequencing, the V3–V5 regions of the 16S rRNA gene were amplified. After confirming that all V3-V5 amplicons had good levels of concentration, purity, and integrity, a massive sequentiation was carried out utilizing the Illumina MiSeq platform (San Diego, California, USA). Reference-based UCLUST algorithm (Qiime1.9.1) was used to pick the OTUs at 97% of similarity against Greengenes v13.8 databases. OTUs were collected in the .biom file and filtered at 0.005% abundance to eliminate spurious OTUs that were present at low frequency.

4.6. Ussing chamber measurements

Intestinal barrier integrity was evaluated in an Ussing chamber system. A segment of small intestine was excised from freshly sacrificed mice and transferred to ice-cold oxygenated Krebs solution containing (mM): NaCl 119, KCl 4.5, $MgSO_4$ 2.5, $NaHCO_3$ 25, KH_2PO_4 1.2, $CaCl_2$ 2.5, glucose 11.1. The segment was cut longitudinally along the mesenteric border and mounted in an Ussing chamber. The Ussing chambers were filled with 10 mL Krebs solution, maintained at 37 °C and continuously bubbled with a hydrated mixture of 5% CO2/95% O2 (v/v). The Ussing chamber system allows to record the short-current (Isc), that is the current generated by the ionic transport through the epithelium. The transepithelial potential difference was continuously monitored under open circuit conditions using a DVC 1000 amplifier (DVC 1000, World Precision Instruments, Sarasota, Florida, USA) and recorded through filled with agar electrodes. The conductance was calculated according to Ohm's law using the potential difference and current (Isc) values. Tissues whose conductance increased during the course of the experiment (calculated every 15 minutes) were considered damaged and excluded from the data analysis.

4.7. Statistical Analyses

Results are shown as means ± the standard error of the mean (SEM). The letter n indicates the number of animals. Statistical analyses were performed using Prism Version 6.0 Software

(Graph Pad Software, Inc., San Diego, CA, USA). The comparison between the groups was performed by ANOVA followed by Bonferroni's post-test. A p-value ≤ 0.05 was considered statistically significant.

5. Conclusions

The present study demonstrates that chronic intake of pistachio exerts beneficial effects in obese mice by alleviating inflammation in adipose tissues and liver and by impacting on the gut microbioma composition. In particular, it enhances the abundance of beneficial bacteria genera such as Lactobacillus, Dorea, Allobaculum and inhibited the growth of bacterial associated with obesity-related comorbidities and inflammation such as Desulfovibrio and Bilophila.

III° ARTICLE

REGULAR INTAKE OF PISTACHIO MITIGATES THE DELETERIOUS EFFECTS OF A HIGH FAT-DIET IN THE BRAIN OF OBESE MICE

Disclosure

The results concerning this article have been published in the Journal *Antioxidants* (Nuzzo et al., 2020).

Summary

The present study investigated the neuroprotective effects of pistachio intake in HFD mice. Three groups of mice were fed a standard diet (STD), HFD, or HFD supplemented with pistachio (HFD-P) for 16 weeks. Metabolic parameters (oxidative stress, apoptosis, and mitochondrial dysfunction) were analyzed by using specific assays and biomarkers. The pistachio diet significantly reduced the serum levels of triglycerides and cholesterol in the HFD model. No difference was observed in the index of insulin resistance between HFD and HFD-P. A higher number of fragmented nuclei were found in HFD cerebral cortex compared to STD and HFD-P. A decrease in reactive oxygen species, singlet oxygen and phosphorylated extracellular signal-regulated kinase, and an increase of superoxide dismutase 2 and heme oxygenase expression were found in the brains of the HFD-P samples compared to HFD. Furthermore, the impaired mitochondrial function found in HFD brain was partially recovered in HFD-P mice. These results suggest that the regular intake of pistachio may be useful in preventing obesity-related neurodegeneration, being able to reduce both metabolic and cellular dysfunctions.

1. Introduction

In the last 50 years, the prevalence of neurodegenerative diseases, including different forms of dementia, is increasing, becoming a social and economic burden. Recent evidence indicates that metabolic dysfunctions may play a key role in the development of neurodegeneration (Anjum et al., 2018; Rojas-Gutierrez et al., 2017).

It is well known that a high-fat diet (HFD) can lead to obesity, type 2 diabetes, non-alcoholic fatty liver disease, and neurodegenerative diseases (Amato et al., 2017; Anjum et al., 2018; Nuzzo et al., 2015; Picone et al., 2011; Rojas-Gutierrez et al., 2017). A correct lifestyle, combining a

healthy diet with regular physical exercise, could prevent metabolic dysfunctions and consequently protect from the related-neurodegenerative disorders.

Natural remedies are currently drawing attention as protective agents in treating obesity-related dysfunctions (Evans et al., 2017; Nuzzo et al., 2018). Food with antioxidant, anti-hyperlipidemia, and anti-inflammatory properties could help to reduce the risk of metabolic dysfunctions (Storz, 2018).

The positive effects of the Mediterranean diet on health have been well documented (Solfrizzi et al., 2011; Vasto et al., 2014a). The longevity of one Mediterranean population, over 90 years old without dementia, was attributed to the nutraceutical components of the Mediterranean diet (Vasto et al., 2014b). Functional food has been reported to delay or inhibit neurodegeneration, suggesting their employment as an alternative therapeutic strategy for correlated diseases (Brown et al., 2015; Carvalho et al., 2018; Nuzzo et al., 2019).

Benefits of nut consumption (mainly pistachios, walnuts, and almonds) have been described in studies on both animals and humans (de Souza RGM et al., 2017; Terzo et al., 2018). Daily nut consumption can improve dysmetabolic conditions such as obesity, type 2 diabetes, and related cardiovascular diseases (Jenkins et al., 2011; Sabatè & Ang, 2009;). In particular, *Pistacia atlantica* oleoresin has been proposed as an agent that protects the body against conditions associated with oxidative stress (Bagheri S et al., 2019), including memory impairment, in lipopolysaccharide-treated rats (Ammari M et al., 2018). Nevertheless, the potential beneficial impact of nut intake on neurodegenerative disorders, as well as on other cognitive-behavioral deficits, has been poorly explored.

Compared to other nuts, pistachios possess a healthier nutritional profile, with low-fat content, high content of polyunsaturated fatty acids (13.3 g/100 g) and mono-unsaturated fatty acids (24.5 g/100 g), minerals (potassium, phosphorus, magnesium, and calcium) and vitamins (vitamin A, vitamin E, vitamin C, and vitamins B). Phytochemicals of pistachio show high bioavailability, contributing to the beneficial relationship between pistachio consumption and health-related outcomes (Mandalari et al., 2013). Furthermore, recent data have demonstrated the ability of pistachio consumption in preventing and ameliorating some obesity-related dysfunctions such as dyslipidemia, hepatic steatosis, and systemic and adipose tissue inflammation (Terzo et al., 2018; Terzo et al., 2020). Accumulation of several lipids associated with an increase in oxidative stress has also been reported in the brain of HFD-fed rodents (Charradi et al., 2017). Lipid dysmetabolism can lead to neuronal damage, causing related-obesity neurodegenerative diseases (Charradi et al., 2017; Lyn-Cook et al., 2009; Mori et al., 2001; Siino et al., 2018). Therefore, we evaluated whether regular pistachio intake has a positive impact, and it exerts beneficial actions in preventing

neurodegeneration induced by HFD in the mouse. For this aim, mice were fed an HFD supplemented with pistachios for 16 weeks, and lipids, oxidative stress, mitochondrial dysfunction, and neurodegeneration were studied in the brain and compared with HFD and standard diet (STD) fed mice.

2. Material and Methods

2.1 Animals, Diets and Experimental Design

Animal experiments were performed in accordance with the Italian legislative decree No. 26/2014 and the European directive 2010/63/UE, and were authorized by the Ministry of Health (Rome, Italy; Authorization no. 349/2016-PR). Four-week-old male C57BL/6J (B6) mice, purchased from Harlan Laboratories (San Pietro al Natisone-Udine, Italy) were housed under standard conditions of light (12 h light: 12 h darkness cycle) and temperature (23 ± 1 ◦C) and relative humidity (55 ± 5%). Food and water were freely available ad libitum.

After one week of acclimatization, the mice were randomly divided into three groups: (a) Mice fed a standard diet (STD, n = 8); (b) Mice fed High Fat Diet (HFD, n = 8); (c) Mice fed an HFD supplemented with pistachio from Valle del Platani, (AG) Sicily, Italy (HFD-P, n = 8). Animals were maintained on each diet for 16 weeks. As previously described [22], the diets supplied were: (1) STD (70% of energy as carbohydrates, 20% protein, and 10% fat; 4RF25, Mucedola, Milan, Italy), (2) HFD (60% of energy as fat, 20% protein, and 20% carbohydrates; PF4215, Mucedola, Milan, Italy), (3) HFD with pistachio (HFD-P; 60% of energy as fat, 20% protein, and 20% carbohydrates; PF4215/C, R&S 34/16, Mucedola, Milan, Italy). HFD-P was custom designed and prepared by Mucedola by substituting 20% of the caloric intake from HFD with pistachio (180 g/kg of HFD). Bodyweight, food intake, and caloric intake were recorded weekly.

At the end of the experimental period, all mice, after fasting overnight, were sacrificed by cervical dislocation. Blood was immediately drawn by cardiac puncture, and plasma was recovered after centrifugation at 3000 rpm at 4 ◦C for 15 min and stored at −80 ◦C until analysis. Then the entire aortic tree was perfused with Dulbecco's phosphate-buffered saline containing 2 mM EDTA. Perfusion was carried out via a cannula introduced into the left ventricle, with incision of the right atrial appendage to permit the outflow of blood. Then, the brains were explanted, washed, weighed, and processed for subsequent analysis. Blood glucose, triglyceride, and cholesterol concentrations were measured by using a glucometer (GlucoMen LX meter, Menarini, Florence, Italy) and Biochemistry Analyzer MultiCare (Biochemical Systems International-Srl, Arezzo, Italy), respectively. Quantification of plasma insulin was carried out by ELISA kit for mouse (Alpco

diagnostics, Salem, NH, USA) according to the manufacturer's instructions and homeostasis model assessment of insulin resistance (HOMA-IR) was calculated.

2.2 Brain Tissue Preparation

Explanted brains from STD, HFD, and HFD-P mice were coronally cut in two halves obtaining an anterior and a posterior part. One part was homogenated in ice by using a Dounce, then separated into aliquots (5 or 10 mg) and immediately flash-frozen in liquid nitrogen and stored until required for analysis. The other part was used for the histological analysis. Thus, the half brain was fixed in 4% formalin for 24 h followed by graded ethanol (50%, 70%, 85%, 96%) for 5 min each, then embedded in paraffin overnight and subsequently sectioned (5 µm thick) using a microtome.

2.3 Tissue Cholesterol Assay

10 mg of frozen homogenate brain tissue was resuspended in 100 µL of PBS and processed using the Amplex red cholesterol assay kit (Life Technology, Monza, Italy), according to the manufacturer's instructions. Absorbance was measured by using the GloMax® Discover multimode plate reader (Promega, Italy) at 490 nm. Cholesterol concentrations were evaluated by using a standard curve, according to the manufacturer's instructions.

2.4 Lipid Peroxidation Assay

To detect the concentration of brain lipid peroxidation, 10 mg of frozen homogenate brain tissue was resuspended in 300 µL of malondialdehyde (MDA) lysis buffer, and the lipid peroxidation MDA assay (Sigma-Aldrich, Milan, Italy) was used according to the manufacturer's instructions. Absorbance was measured at 532 nm by using the GloMax® Discover multimode plate reader. To detect the concentration of plasma lipid peroxidation, 20 µL of plasma was processed as described above.

2.5 Detection of Oxidative Levels: DCFH-DA Assay

Reactive oxygen species (ROS) generation in the brain was evaluated by using 2',7'-dichlorofluorescin diacetate (DCFH-DA; Molecular Probes, Eugene, OR, USA). 5 mg of frozen homogenate brain tissue was resuspended in 5 mL of PBS buffer. After centrifugation, 100 µL of the supernatant was plated and incubated for 5 min with 1 µL of DCFH-DA (1 mM). Oxidation levels were evaluated using the GloMax® Discover system (Promega) at 37 ◦C at an excitation wavelength of 475 nm and an emission wavelength of 555 nm. To evaluate the presence of ROS in the plasma 1 µL of DCFH-DA (1 mM) was added to 20 µL of plasma and processed as described

above.

2.6 TUNEL Assay

Apoptosis was evaluated by using the in situ cell death detection kit, TMR red (Roche, Monza, Italy) according to the manufacturer's instructions. Briefly, after progressive hydration, sections of paraffin-embedded brains (5 µm thick) were incubated with permeabilization solution for 8 min, washed in PBS, and incubated with TUNEL reaction mixture for 60 min at +37 ◦C in a humidified atmosphere in the dark. After washing in PBS, the slides were incubated with Hoechst 33258 (5 µg/mL) for 20 min and analyzed by using a DHL fluorescent microscope (Leica Microsystems, Heidelberg, Germany) at a magnification of 20×.

2.7 Lipid Nile Red Staining

To evaluate the presence of lipids in the brain tissue, the deparaffinized brain sections from STD, HFD, and HFD-P mice were hydrated in graded ethanol for 5 min each. After washing in PBS, the sections were stained by using Nile Red (0.5 µL/mL) (ThermoFisher Scientific, San Jose, CA, USA) at room temperature for 1 h. Samples were analyzed by using a DHL fluorescent microscope.

To quantify lipids in the brain tissue, 10 mg of frozen brain homogenate were resuspended in 1 mL of PBS and Nile Red (0.5 µL/mL). The homogenate was incubated at room temperature for 15 min. 2 µL of the solution was spotted onto a nitrocellulose membrane, and the fluorescence was visualized by using the Typhoon FLA 9500 scanner (excitation/emission 552/636 nm). The images were analyzed by ImageQuant TL software (GE Healthcare Life, Marlborough, MA, USA).

2.8 Singlet Oxygen ($^1O_2$) Species Generation

10 mg of frozen homogenate brain tissue was resuspended in 1 mL of PBS; the samples were centrifuged at 10,000 g at 4 ◦C for 10 min. 48 µL of the supernatant of each sample was mixed with 50 µL of the reagent buffer and 2 µL of 5 mM SOSG agent (Molecular Probes). 80 µL of each sample mixture was added into a well of 96-well plate (Black Microtiter Plate, Thermo Scientific, Vantaa, Finland) and covered with transparent lid. The fluorescence signal was measured at excitation 488 nm and emission 525 nm by a spectrophotometer GloMax® Discover system.

2.9 SOD Activity Levels

10 mg of frozen homogenate brain tissue was resuspended in 1 mL of PBS buffer with protease inhibitors (Amersham Life Science, Munich, Germany). To remove insoluble material,

tissue lysates were sonicated on ice (cooled for 30 s and sonicated for 30 s twice at low power output, 20 W). After centrifugation (14,000 rpm, at 4 ◦C, for 30 min), the supernatant was submitted to the Bradford method for protein quantification. A volume corresponding to 50 µg of total proteins was used for SOD enzymatic activity measurement, by using the SOD assay kit (Sigma-Aldrich) according to the manufacturer's instructions. Absorbance was measured at 450 nm by using the GloMax® Discover multimode plate reader.

2.10 *Immunofluorescence Analysis*

Coronal brain sections were mounted on slides and deparaffinized in xylene and hydrated in a series of graded ethanol (96%, 85%, 70%, 50%) for 5 min each. The slides were incubated at 4 ◦C overnight with the primary antibody anti-phosphorylated extracellular signal-regulated kinase (p-ERK; 1:25; Santa Cruz Biotechnology, Heidelberg, Germany) and anti-microtubule-associated protein light chain 3 (LC3; 1:25; Santa Cruz Biotechnology). After washing in PBS, the slides were incubated with anti-rabbit Cy3-conjugate secondary antibodies (1:500; Cell Signaling Technology, Danvers, MA, USA). Nuclear staining was performed using Hoechst 33258 (5 µg/mL) for 20 min. The slides were analyzed by using a DHL fluorescent microscope (Leica Microsystems, Heidelberg, Germany) at a magnification of 20×. p-ERK and LC3 positive fluorescence intensity were measured by using a Leica QFluoro program (Leica Biosystems, Wetzlar, Germany). The immunohistochemical staining was run in triplicates per mouse for each antibody and observed by two independent research in four slides.

2.11 Total Protein Extraction and Western Blot

Total proteins were prepared by resuspending 10 mg of frozen homogenate in solubilizing buffer (50 mM Tris-HCl, pH 7.4; 150 mM NaCl, 0.5% Triton X-100, 2 mM PMSF, 1 mM DTT, 0.1% SDS) with protease inhibitor (Amersham, Life Science, Les Ulis, France) and phosphatase inhibitor cocktail II (Sigma-Aldrich, Poole, Dorset, UK). Total proteins were quantified by the Bradford method (Bio-Rad, Segrate, Italy). 50 µg of protein samples were resolved by 12% acrylamide gel and transferred onto a nitrocellulose filter for Western blotting. The filter was incubated with anti-superoxide dismutase 2 (SOD2; 1:500, Santa Cruz Biotechnology), anti-heme oxygenase (H-Oxy; 1:1000, Cell Signaling Technology), anti-heat shock protein 60 (HSP60; 1:500, Cell Signaling Technology), anti-mitochondrial dynamin-like GTPase 1 (OPA1; 1:500, Santa Cruz Biotechnology), anti-dynamin-related protein 1 (DRP1; 1:500, Santa Cruz Biotechnology), anti-mitochondrial fission 1 protein (FIS1; 1:500, Santa Cruz Biotechnology), anti-PTEN-induced kinase 1 (Pink1; 1:500, Santa Cruz Biotechnology), anti-RBR E3 Ubiquitin Protein Ligase (Parkin;

1:500, Santa Cruz Biotechnology), anti-ubiquitin-binding protein p62 (p62; 1:500, Santa Cruz Biotechnology), and anti-β-actin (β-Actin; 1:10,000, Sigma-Aldrich). Primary antibodies were detected using the Odyssey® scanner (LI-COR Biosciences, Lincoln, NE, USA), according to the manufacturer's instructions, using secondary antibodies (anti-mouse and anti-rabbit) labeled with IR790 and IR680 (1: 10,000; Life Technology). Band intensities were analyzed with the Odyssey® CLx imaging system, and expression was adjusted to actin expression. The protein levels were expressed as intensity relative to control.

2.12 Isolation of Brain Mitochondria

Cytosol and mitochondria fractions from 10 mg of frozen brain tissue, were prepared using Mitochondrial isolation kit (ThermoFischer, Italy) according to the manufacturer's instructions using buffers provided by the kit. Briefly, the brain homogenate (10 mg) was resuspended in 200 µL of lysis buffer, centrifuged at 2000 g for 3 min to remove cell debris. The supernatant was centrifuged at 10,000g for 5 min and the mitochondrial pellet was washed twice by centrifugation at 10,000g for 10 min and resuspended in the buffer provided by the kit. An aliquot was used to determine protein concentration (2 µg/µL) by the Bradford method. The amount of mitochondrial protein is usually accepted as a mitochondrial quantity (Chapa-Dubocq et al., 2018), and an equal amount (50 µg) of mitochondrial protein was used for each measurement in all experiments.

2.13 Mitochondrial Stress

The presence of superoxide in brain isolated mitochondria was analyzed by fluorescence using the MitoSOX Red reagent (Molecular probes, Paisley, UK). A volume corresponding to 50 µg of mitochondrial proteins was incubated with MitoSOX reagent (5 µM) for 10 min at 37 ◦C in the dark. At the end of the incubation, the solution was centrifuged at 10,000g for 5 min, and the mitochondrial pellet was resuspended in PBS and analyzed by GloMax® Discover multimode plate reader (Promega, Italy) at the excitation wavelength of 514 nm and to record the emission spectrum in the range 540–640 nm. A dilution of the sample (1:50) was used for microscopic inspection (DHL fluorescent microscope Leica Microsystems, Heidelberg, Germany).

2.14 Mitochondrial Swelling

The swelling of brain isolated mitochondria was evaluated according to Chapa-Dubocq et al. [27], by measuring the changes in the absorbance of the mitochondrial suspensions at 540 nm using a GloMax® Discover multimode plate reader (Promega, Italy). A volume corresponding to 50 µg of mitochondrial proteins was incubated with 50 µL of buffer (125 mM KCl, 1 mM $MgCl_2$, 5 mM

malate, 5 mM glutamate, 1 µM EGTA, and 20 mM Tris base) at pH 7.4. The absorbance was monitored for 5 min at 37 ◦C at 540 nm, and the mitochondrial swelling was indicated by a decrease in the absorbance at 540 nm.

2.15 *Statistical Analysis*

The results are presented as mean ± SEM. A one-way ANOVA was performed, followed by Dunnett's post hoc test for analysis of significance. Results with a p-value < 0.05 were considered statistically significant.

3. Results

3.1 Effects of Pistachio Intake on Metabolic Parameters

As shown in Figure 1A, HFD and HFD-P mice presented a body weight significantly higher than the lean mice. No difference in the daily food intake was observed among the three different groups (Figure 1B). The net energy intake in HFD and HFD-P animals was higher than lean mice (Figure 1C).

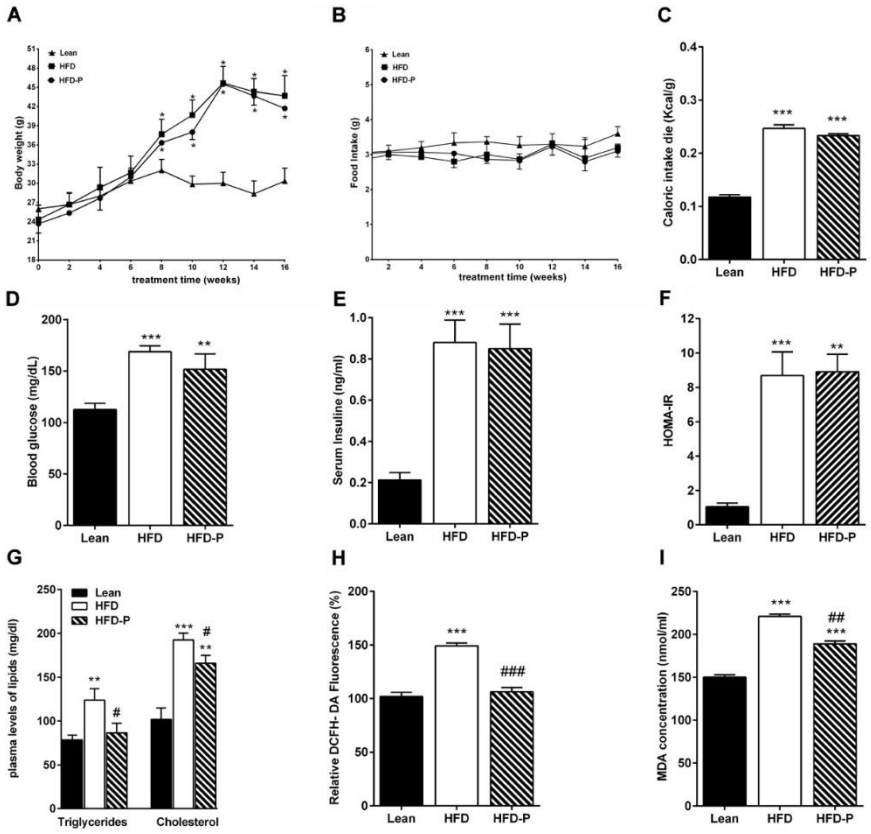

Figure 1. Pistachio consumption attenuates alterations of various metabolic and oxidative parameters in HFD mice. Values in mice after 16 weeks of standard diet (STD), HFD or HFD supplemented with pistachio (HFD-P): A) Body weight; B) Daily food intake; C) Caloric intake; D) Fasting glucose concentration; E) Serum insulin concentration; F) HOMA-IR; G) Plasma levels of triglycerides and cholesterol; H) Plasma ROS levels in STD, HFD or HFD-P mice expressed as % of STD; I) Plasma lipid peroxidation levels in the different animal groups. Data are means ± S.E.M. (n = 8/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* STD. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs* HFD.

Systemic metabolic parameter analysis showed that HFD and HFD-P fasting glycemia, insulin concentration, and HOMA index were more elevated than lean mice, indicating an impairment in glucose metabolism, which was not improved by pistachio consumption (Figure 1D, F). However, regular pistachio intake significantly reduced the HFD-increased serum levels of triglycerides and cholesterol (Figure 1G). In addition, we observed significantly higher ROS and peroxidation lipid levels in HFD plasma than lean. These increases were attenuated in HFD-P plasma (Figure 1H, I).

3.2 Effects of Pistachio Intake on Neurodegeneration

To understand whether regular pistachio intake can reduce the risk of neurodegeneration, the brain was weighed at the time of the sacrifice. Slight differences in brain weight were observed among the three groups. However, a significant reduction of the brain/body weight ratio was found in the HFD group in comparison with STD. This reduction was less pronounced in HFD-P (Table 1). Moreover, a significantly increased number of fragmented nuclei was found in the cerebral cortex of HFD mice compared to STD and HPD-P mice, suggesting that pistachio consumption can counteract neurodegeneration (Figure 2A–C).

Table 1. Effects of pistachio diet on body weight and brain weight. HFD-P showed significantly increased brain/body weight ratio in comparison with HFD, suggesting a preventive action of pistachio consumption against brain atrophy.

Diet	Mouse C57BL/6	Age (months)	Body weight (g) (± SEM)	p-value	Brain weight (g) (± SEM)	p-value	Weight ratio Brain/body	p-value
STD	8	4	30.4 ± 0.09		0.31 ± 0.09		0.01019 ± 0.002	
HFD	8	4	44.2 ± 0.02*	< 0.005	0.28 ± 0.06*	< 0.05	0.00633 ± 0.001*	< 0.05
HFD-P	8	4	42.1 ± 2*		0.30 ± 0.05*	< 0.05	0.00712 ± 0.002#	< 0.05

STD: standard diet, HFD: High-Fat diet; HFD-P: High Fat Diet supplemented with pistachio; SEM: standard error medium; * denotes significant difference compared with the STD; # denotes significant difference compared with the HFD group.

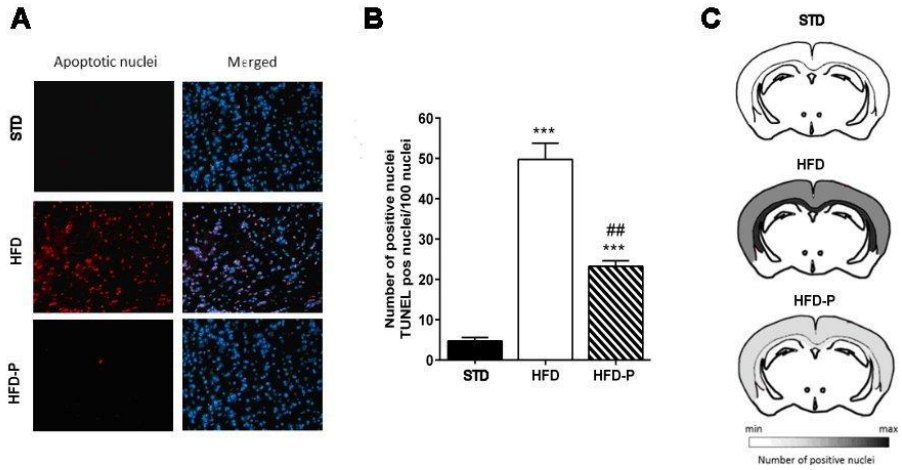

Figure 2. Pistachio consumption exerts a neuroprotective effect. A) TUNEL assay on cerebral cortex sections of STD, HFD and HFD-P mice; B) Number of apoptotic nuclei in the cerebral cortex; C) Scheme of distribution of positive TUNEL nuclei. Data are means ± S.E.M. (n = 8/group). *** $p < 0.001$ *vs* STD, ## $p < 0.01$ *vs* HFD.

3.3 Regular Pistachio Consumption Improves HFD-Induced Lipid Dysmetabolism in the Brain

Since obesity and aberration of lipid homeostasis are often linked to neurodegenerative disorders (Charradi et al., 2017; Lyn-Cook et al., 2009), we analyzed the brain cholesterol and lipid content in the different animal groups. Significantly higher levels of cholesterol were found in the HFD brain in comparison with STD and HFD-P brain (Figure 3A). Furthermore, large and homogeneous distribution of lipids in HFD coronal sections was observed, while in the HFD-P brain, it was less and more similar to the STD brain (Figure 3B, C). To quantify the cerebral lipids, the whole brain homogenate was stained with Nile Red. The fluorescence intensity, which was significantly increased in the HFD brain, was less in the HFD-P brain (Figure 3D, E).

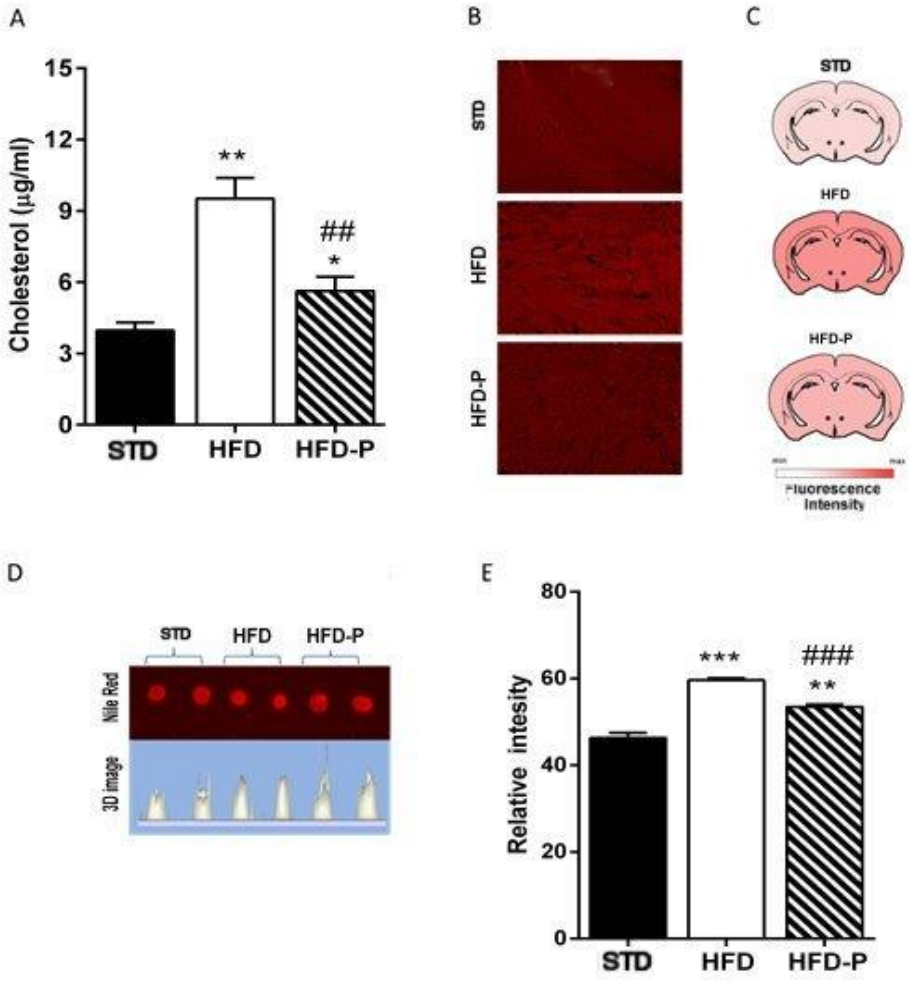

Figure 3. Pistachio consumption prevents HFD-induced brain lipid accumulation. A) Cholesterol concentration in brain tissue of STD, HFD and HFD-P fed mice; B) Lipids content measured by Nile Red staining in coronal brain sections of STD, HFD and HFD-P mice; C) Scheme of distribution of fluorescence after Nile Red staining; D) Fluorescence in brain lysates of STD, HFD and HFD-P mice after Nile Red staining; E) Quantification of Nile Red staining fluorescence. Data are means ± S.E.M. (n = 8/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ vs STD. ## $p < 0.01$, ### $p < 0.001$ vs HFD.

3.4 Pistachio Reduces Oxidative Stress in the Brain of HFD Mice

Lipid dysregulation is linked to brain metabolic stress, which is a risk factor for the neurodegeneration (Anjum et al., 2018). For this reason, we verified the stress conditions in the brain. By ROS and $^1O_2$ assays, we observed high levels of ROS in the brain of HFD mice that were partially counteracted by a diet with pistachios (Figure 4A, B). Accordingly, reduced lipid peroxidation was found in the brain of HFD-P mice (Figure 4C). Furthermore, the immunoreactivity of p-ERK, a typical stress marker, was found exclusively in sections of the HFD cerebral cortex and not in other brain regions such as the hippocampus, thalamus, and hypothalamus.

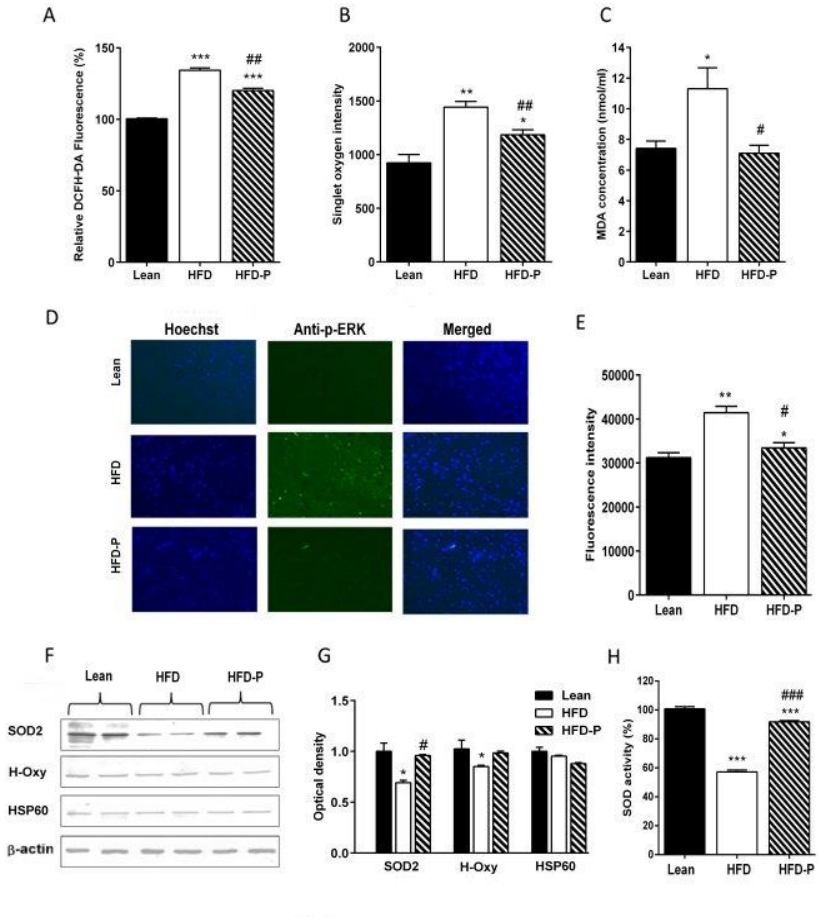

Figure 4. Pistachio consumption prevents HFD-induced brain ooxidative stress. A) Levels of ROS in brain tissue of STD, HFD or HFD-P fed mice measured by DCFH-DA assay (% respect to STD); B)$^1O_2$ intensity; C) Lipid peroxidation levels in brain tissues of STD, HFD or HFD-P fed mice measured by MDA assay; D) Immunofluorescence of cerebral cortex sections of STD, HFD and HFD-P mice incubated with anti-phosho-ERK; E) Quantification of p-ERK immunofluorescence; F) Western blot of proteins extracted from STD, HFD, HFD-P brain lysates and incubated with anti-SOD2, anti-H-Oxy and anti-HSP60. Uniformity of gel loading was confirmed by β-actin as standard; G) Densitometric analysis of immunoreactivity; H) Total SOD2 activity levels expressed as % respect to STD in each tissue extract. Data are means ± S.E.M. (n = 8/group). Asterisk denotes significant difference compared with the STD group (* $p < 0.05$, HFD *vs* STD); hash denotes significant difference compared with the HFD group (# $p < 0.05$, HFD-P *vs* HFD).

We also observed a less immunoreactivity of p-ERK in the HFD-P group (Figure 4D, E) and downregulation of SOD2 and H-Oxy in the brain of HFD mice (Figure 4F,G). In contrast, HFD-P mice showed a level of expression similar to lean for both proteins (Figure 4F, G). No significant difference in HSP60 levels of expression was observed. Instead, decreased SOD2 activity was found in the HFD group as compared to the lean or HFD-P group (Figure 4H).

3.5 Pistachio Regular Intake Maintains Mitochondrial Homeostasis

Mitochondrial dysfunction is a consequence of oxidative stress, and it is caused by different factors, including impairment of the dynamics.

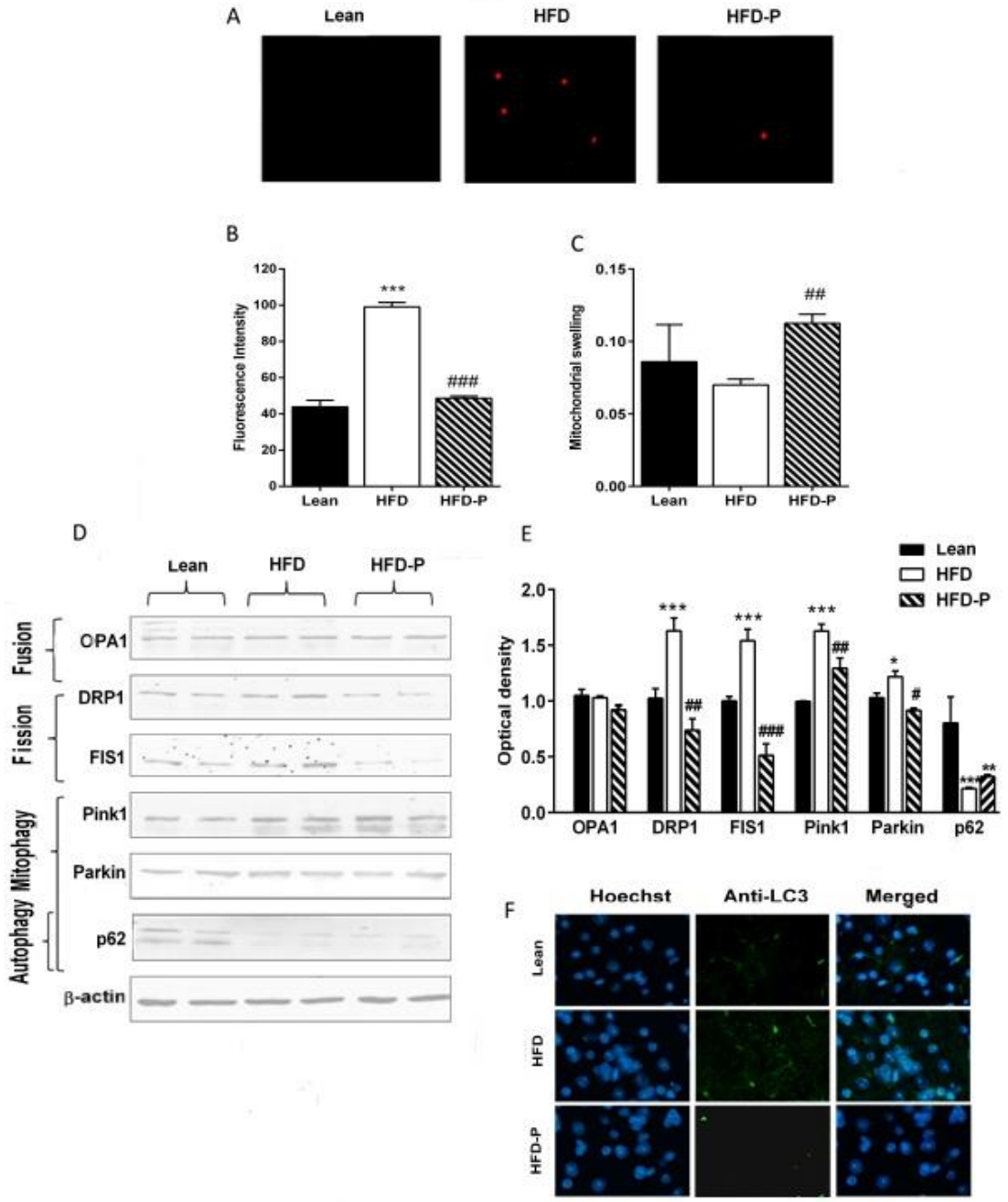

Figure 5. Pistachio intake counteracts HFD-induced mitochondrial dysfunction. A) Mitochondrial stress in enriched mitochondria fraction from Lean, HFD or HFD-P fed mice by MitoSox staining; B) Level of fluorescence intensity by MitoSox assay; C) Mitochondria swelling in STD, HFD and HFD-P brains; D) Western blot of proteins extracted from brains of Lean, HFD or HFD-P mice and incubated with antibodies against proteins involved in mitochondrial dynamics (OPA1, DRP1, FIS1), and mitophagy (PINk1, Parkin, p62). Uniformity of gel loading was confirmed with β-actin as standard. E) Densitometric analysis; F) Immunofluorescence of cerebral cortex sections of STD, HFD or HFD-P mice incubated with anti-LC3. Data are the means ± S.E.M. (n = 8/group). * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ *vs* STD. # $p < 0.05$, ## $p < 0.01$, ### $p < 0.001$ *vs* HFD.

After mitochondria separation, MitoSox assay was used to identify mitochondrial superoxide selectively, and the swelling test was performed as an indicator of permeability transition pore (PTP). High levels of oxidative species inside the mitochondria and increased swelling were found in HFD mice. These conditions were attenuated by a diet, including pistachio (Figure 5A–C).

Furthermore, expression of proteins involved in fission and fusion events and ubiquitin-dependent mitophagy were analyzed. No significant difference in OPA1, a protein involved in the fusion process, was observed among the three different groups. In contrast, DRP1 and FIS1, proteins involved in the fission process, were more expressed in the HFD group, than HFD-P fed mice. In the HFD brain, the expression of Pink1, a mitochondrial damage sensor, and Parkin, a signal amplifier, was increased. In contrast, the expression of p62, the signal effector, was decreased, suggesting the presence of damaged mitochondria, a condition partially recovered by HFD-P (Figure 5D,E). Finally, the accumulation of LC3 in HFD coronal brain sections was observed by immunofluorescence assay, confirming the increase of mitophagy and lysosomal activity compared to lean. At the same time, LC3 was less expressed in the HFD-P samples (Figure 5F).

4. Discussion

The present study provides experimental evidence for the beneficial neuroprotective effects of regular pistachio intake in the brain of obese mice. This preventive action takes place through the reduction of lipid dysmetabolism, oxidative stress, and mitochondrial dysfunction.

It is now widely accepted that the consumption of HFD is a risk factor for the development of obesity-related diseases and prolonged HFD feeding has been reported to accelerate the pathogenesis of neurodegeneration (Keshk et al., 2020; Nuzzo et al., 2015; Stranahan et al., 2008), leading to impairment of cognitive functions in rodents (Stranahan et al., 2008).

Although an increasing number of experimental and clinical observations have led to consider almonds, hazelnuts, and walnut as brain-protective agents, mainly against brain atrophy, memory loss, and Alzheimer's disease (Gorji et al., 2018), few studies can be found about pistachio. Indeed, Singh et al. (Singh S & Dharamveer Kulshreshtha M, 2019) demonstrated that *Pistacia vera* fruit extracts improve mouse cognitive processes after chemically-induced deficits; however, the mechanisms responsible have not been clarified yet. Pistachio regular consumption has already been shown to enhance the obesity-related dysfunctions, including inflammation, by positively modulating the expression of genes linked to the lipid metabolism (Amato et al., 2017; Terzo et al., 2018). Therefore, the goal of the present study was to verify whether supplementing an HFD with pistachio fruits can mitigate the harmful effects associated with the consumption of an HFD in the brain of obese mice.

The potential beneficial effect of pistachio on neurodegeneration was investigated by using a HFD fed mouse that provided a suitable model for studies on diet-induced metabolic changes and redox equilibrium disorders (Nuzzo et al., 2015). First, we determined if supplementation of an HFD with pistachio affected the neurodegeneration already showed to be present in the mouse

cerebral cortex after 8 weeks on HFD (Nuzzo et al., 2019). We found a neuroprotective action of pistachio consumption, which partly prevented HFD-induced neuronal apoptosis, as demonstrated by a reduced number of cells with fragmented DNA in the cortical areas. Indeed, as we previously reported, the brain dysfunctions in long term HFD fed mice are associated with peripheral and central insulin resistance (Nuzzo et al., 2015) and dyslipidemia (Nuzzo et al., 2018). For this reason, we verified the hypothesis that regular pistachio intake could influence glucose or lipid metabolism. The results obtained by measuring the metabolic parameters allow us to discard the supposition that the effects of pistachio consumption in the brain are a direct consequence of actions on glucose metabolism or bodyweight, because no changes in fasting glycemia, HOMA index, or body weight were observed. It was noted that the inability of chronic pistachio intake to change the bodyweight or influencing glucose dysmetabolism was already reported both in humans and animal models (Gulati et al., 2014; Terzo et al., 2018). Our results suggest that the effects of regular pistachio intake are attributable to beneficial actions on lipid dysmetabolism and redox state. This is in agreement with previous studies on animals (Alturfan et al., 2009; Aksoy et al., 2007; Terzo et al., 2018) and humans (Kocyigit et al., 2006; Sari et al., 2010), where pistachio intake was able to decrease the HFD-induced high levels of plasma cholesterol, triglycerides, and oxidative stress, suggesting lipid-lowering and antioxidant properties of pistachio fruit. Thus, we cannot exclude that the antioxidant content of pistachio can activate a compensatory mechanism for mainly mitigating HFD-induced systemic and central dysmetabolism and redox stress.

An altered lipid metabolism is believed to be a critical event that contributes to central nervous system injuries (Liu et al., 2010). In our experiments, HFD induced high cholesterol and neutral lipid levels in the brain. Hyperlipidemia and more exactly high cholesterol levels have been reported in the brains of patients with Alzheimer's disease (Mori et al., 2001), and they have been shown to worsen brain injury in an experimental mouse model (ElAli et al., 2011). A recent study reported that increased free cholesterol induces neuronal death via endoplasmic reticulum stress and activation of apoptotic mechanisms (Djelti et al., 2015). Noteworthy, the pistachio anti-lipotoxic effect was also found in the brain, as evidenced by the reduction in lipids and oxidative stress, suggesting a beneficial protective action against neuronal damage. Of note is that the oxidative lipids and ceramides, increased by systemic dysmetabolism, are able to pass through the blood-brain barrier, and they can also contribute to the oxidative dysmetabolism that is already occurring in the brain, accelerating the progression of the neurodegeneration (Lyn-Cook et al., 2009; Nuzzo et al., 2018).

In agreement with these findings, our results indicate that the antioxidant activity of pistachio improves dysfunctional of nervous system damage, including neurodegenerative disorders

(Ali et al., 2015). Indeed, the cerebral tissue is among the most vulnerable tissues to oxidative stress because of its high levels of polyunsaturated fatty acids that are susceptible to lipid peroxidation. Moreover, it also has lower antioxidant defences in comparison with other organs (Dringen, 2000; Uttara et al., 2009;). Fatty acid oxidation produces ROS, which can induce neurodegeneration via apoptotic pathways (Kritis et al., 2015; Mehta et al., 2013;). The HFD model is clearly lipotoxic due to the high ROS and lipoperoxidation levels, the increased expression of p-ERK, and down-regulation of H-Oxy and SOD2 that we found in HFD brains. In agreement with previous reports (Ruegsegger et al., 2019), the activity of the SOD2 was lower in the HFD brain, suggesting that reduced antioxidant defense may contribute to the increased oxidative stress. However, the changes in the stress markers were less pronounced in the HFD-P brain, confirming the antioxidant activity of the pistachio, already demonstrated in other HFD tissues (Terzo et al., 2018). These properties could be related to the presence of phytosterols (stigmasterol and campesterol), lutein (xanthophyll carotenoid), and polyphenols (resveratrol and catechins) (D'Evoli et al., 2015; Terzo et al., 2019).

Impaired mitochondrial function is one factor that contributes to degenerative brain disorders (Lin & Beal, 2006), and the mitochondrial dysfunction has been shown to be the primary cause of the cellular apoptosis (Desagher & Martinou, 2000). We found significantly increased brain mitochondrial stress and swelling after long-term HFD, indicative of mitochondrial dysfunction (Sa-Nguanmoo et al., 2017). Mitochondrial swelling is the endpoint of a cascade of events induced by excessive ROS generation and including altered Ca^{2+} uptake and PTP opening. A balance between fission and fusion of mitochondria is critical for mitochondrial functional integrity, and an increase of fission events can produce accumulation of damaged mitochondria that can be removed by selective mitophagy. In our experimental conditions, HFD disrupts the mitochondrial homeostasis, as demonstrated by overexpression of DRP1 and FIS1, proteins involved in the fission process, and by the changes in the expression of Pink1, parkin, and p62, proteins involved in the pathway that regulates ubiquitin-dependent mitophagy. Moreover, HFD induces autophagy-related processes, as suggested by LC3 increased expression.

Interestingly, regular pistachio intake seems to counteract these adverse effects of HFD by preventing the mitochondrial brain dysfunction and autophagosome-lysosome fusion, suggesting once more the beneficial effects of pistachio fruit consumption on brain health. In agreement with our results, several findings indicate that antioxidants of pistachio fruit, including phytosterols and polyphenols, influence mitochondrial dynamics by maintaining the balance between fusion and fission and preventing mitophagy. In addition, modification of autophagy by polyphenols has been proposed as a promising therapeutic strategy (Naoi et al., 2019).

Further, we have to consider that selective mitophagy leads to mitochondrion number reduction with a consequent decrease in ATP production, as well as reduced metabolic activity in the brain. Preclinical and clinical studies have demonstrated that a diet supplemented with antioxidants and combined with exercise training can stimulate mitochondrial biogenesis, a mechanism that can replace the loss of damaged mitochondria (Mankowski et al., 2015; Steiner et al., 2011). Thus, a diet, including pistachio, especially in combination with exercise training, can represent an efficient strategy to improve dysmetabolism and delay neurodegeneration.

In conclusion, our results demonstrate that pistachio consumption has beneficial effects against the negative impact induced by long-term HFD in the mouse brain by exerting neuroprotective activities. The neuroprotective effects include decreased brain apoptosis, decreased brain lipid, and oxidative stress with the associated improvement of mitochondrial function. In particular, regular pistachio intake attenuates mitochondrial ROS generation induced by a high-fat diet, which in turn reduces damaged mitochondria (Fig. 6), leading to beneficial effects on mitochondrial dynamics and mitophagy.

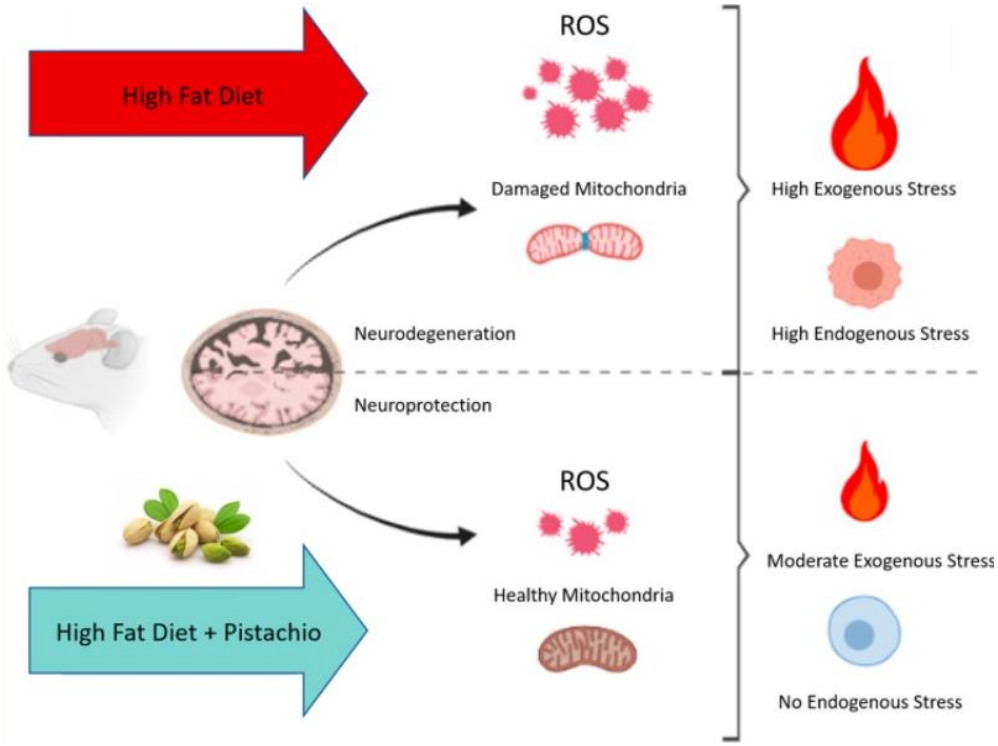

Figure 6. Schematic representation of the neuroprotective effect of regular pistachio intake.

GENERAL DISCUSSION

The results presented in this thesis have been discussed within each article. In this chapter, I will try to underline the more original and less developed aspects.

Taken together, our results have provided evidence for beneficial effects of the pistachio regular consumption, being able to prevent various obesity-related dysfunctions in HFD obese mice. Although we examined the impact of pistachio intake in an animal model (HFD obese mouse), the pistachios could represent potentially a functional food in preventing obesity-related metabolic dysfunctions. Pistachios are a rich source of mono- and polyunsaturated fatty acids, phytosterols and phenols with antioxidant and anti-inflammatory properties, they are cholesterol-free and have low saturated/polyunsaturated fatty acid ratio and low glycemic index.

In according to previous human studies addressed to evaluate the effects of pistachio consumptions on lipd profile (Kocyigit et al., 2006), or glucose metabolism (Ribeiro et al., 2019), we used a daily dose equivalent to 20% of the total daily calorie intake, corresponding to about 65 - 75 g/day in adults. Therefore, it seems possible to consider the pistachio dose used in our study as "functional" to be included in the human daily diet, for example ingested as a snack. However, our result cannot rule out the hypothesis that lower daily pistachio intake is also efficacious in preventing the obesity-related dysfunction. Further studies should be necessary to clarify this point.

Different previous studies on rodents or humans reported beneficial effects of pistachio consumption on various aspects of the metabolic syndrome (Sauder KA et al., 2014; West SG et al., 2012; Papada E et al., 2018; Hernández-Alonso P et al., 2014; Assaf-Balut C et al., 2017). In particular, evidences were provided for lipid lowering properties and improvement of plasma dyslipidemia (Alturfan et al., 2009; Aksoy et al., 2007; Gebauer et al., 2008; Kocyigit A; Sari et al., 2010; Holligan et al., 2014; Hernández-Alonso et al., 2015). However, different mechanisms have been proposed to be responsible for the hypolipidemic effects of pistachio consumption. They could be caused by the high content in MUFA and PUFA (Silva Figueiredo et al., 2017) and/or phytosterols (Kornsteiner-Krenn M et al., 2013), that inhibit cholesterol intestinal absorption (Altmann et al., 2004). Our experiments for the first time pointed out a new mechanism by which the regular pistachio intake can exert beneficial effects on lipid metabolism. In fact, pistachio regular intake modulated positively the expression of lipid metabolism-related genes in liver and adipose tissue. Indeed, our results showed *PPAR-γ* and *SCD1*, transcription factors of genes involved in lipid metabolism, as the main targets for the preventive action of pistachio consumption. *PPAR-γ* and *SCD1*expressions were significantly reduced both in the liver and adipose tissue of HFD-P mice in comparison with HFD mice, suggesting pistachio ability to inhibit *de novo* lipogenesis. Moreover, we observed in adipose tissue, a reduction in the expression of

SREBP-1c, a master regulator of fatty acids synthesis, (Crewe C et al., 2019) and FAT-P which is involved in the fatty acid uptake from the extracellular milieu (Jia et al., 2007) suggesting pistachio ability in reducing fatty acid transport in adipose tissue. These changes in gene expression could account for the improvement of steatosis, decrease in hepatic lipids, in adipocyte size and in total visceral fat mass observed in HFD-P mice compared to HFD animals.

Indeed, the results from our experiments showed for the first time that pistachio consumption exerts preventive and improving effects on hepatic steatosis and fat liver accumulation. In fact, histological analysis of hepatic tissue pointed out that the steatosis degree, very severe in HFD mice, was significantly improved in HFD-P mice. In agreement with the morphology improvements, a significant decrease in liver intrahepatic lipids was observed in HFD-P animals in comparison with HFD group. Oxidative stress is an important mechanism in the pathogenesis of hepatic steatosis (Su et al., 2016). Some studies have shown that high ROS levels modify the redox conditions of the liver cells and oxidative stress alters lipid, protein, and DNA molecules and triggers inflammatory signalling pathways, which promote steatosis progression (Cichoż-Lach H & Michalak A, 2014; Pierantonelli I &Svegliati-Baroni G, 2019). Although we did not evaluate the hepatic oxidative stress in the different animal groups, we found a reduction in the Il-1β and CCL-2 mRNA expression and a decreased number of inflammatory foci in HFD-P liver in comparison with HFD mice, suggesting that regular pistachio consumption is able to slow down the progression of NAFDL induced by HFD.

Furthermore, our experiments pointed out various changes also in the adipose tissue of HFD-P in comparison with HFD. Adipose tissue is the first tissue affected by excessive lipids intake. The mechanisms by which adipose tissue expands itself in response to an excessive caloric intake represent a crucial determinant for the metabolic dysfunction risk. The expansion is mediated by an increase in adipocyte numbers (hyperplasia) and/or an enlargement of adipocyte size (hypertrophy). Hyperplasia allows a “healthy” expansion of the adipose tissue, because it is due to the formation of functional adipocytes from progenitor cells (adipogenesis). In contrast, adipocyte hypertrophy typically leads to lipid-laden, dysfunctional adipocytes that undergo cell death and contribute to adipose tissue inflammation, dysfunction and associated pathologies (Fuster JJ et al., 2016). Indeed, adipocyte hypertrophy is associated with increased adipokine and pro-inflammatory cytokine production (Skurk et al., 2007) and leads to hypoxia and adipocyte cell death (Giordano et al., 2013). Dysfunctional VAT undergoes to accumulation of inflammatory cells; in particular, active macrophages infiltrate VAT and surround dead adipocytes in typical “crown-like structures” (Wang et al., 2005).

Our study suggested that pistachio chronic intake reduces the hypertrophy in adipose tissue because adipocyte diameter and area were significantly reduced in HFD-P mice in comparison with HFD mice. In according to this beneficial effect, RT-PCR and immunohistochemistry analysis revealed the pistachio ability to counteract adipose tissue inflammation. In fact, we found down-regulation of TNF-α, F4-80 and CCL-2, as well as reduced density of crown-like structures, index of minor macrophage infiltration, in the adipose tissue of HFD-P mice compared to obese control group suggesting that pistachio-based diet is able to affect the mechanisms leading to increased infiltration of macrophages into VAT.

Pistachio regular consumption ameliorated not only hepatic and adipose tissue inflammation but also the systemic inflammation, because it prevented the increase of the pro-inflammatory cytokines, TNF-α and IL-1β, induced by HFD in the blood. However, our experiments do not allow us to understand if the pistachio anti-inflammatory action is the consequence of the reduced expansion and dysfunction of VAT, usually triggering the vicious cycle between adipocytes and macrophages resulting in chronic low grade inflammation or it is due to flavones and anthocyanins content. In fact, recently, polyphenol extracts from pistachios have been showed to possess anti-inflammatory properties, in both *in vitro* (monocyte/macrophage cell line with LPS-induced inflammation) and *in vivo* (rat paw edema induced by carrageenan injection) models (Paterniti I et al., 2017).

It is well known that low-grade of inflammation is a link between obesity and insulin resistance (Wu & Ballantyne, 2020). Although, we pointed out anti-inflammatory properties of regular pistachio intake, we did not find beneficial effects on glucose dysmetabolism. In our experiments, pistachio intake did not affect HFD-induced hyperglycemia or insulin resistance index. Our data are in agreement with a study reporting no significant change in insulin concentrations or in fasting plasma glucose during the pistachio-enriched diet period in subjects with metabolic syndrome (Wang X et al., 2012), but they are in contrast with other data in literature (Parham M et al., 2014; Feng X et al., 2019; Ribeiro PVM et al., 2019). The discrepancy makes difficult at this stage to reach definite conclusions on pistachio impact on glycaemic control over the long term.

Interesting results were also obtained by micro-computed tomography. It revelead significantly decreased VAT and increased SAT volume in HFD-P mice compared to HFD mice, suggesting that pistachio regular consumption could be responsible of an adipose tissue redistribution linked to a healthier profile. In fact, VAT increase has been reported to be strictly associated with cardiometabolic risk. On the contrary, SAT accumulation is retained to be protective, relatively to visceral fat, because SAT takes up lipid from circulation and stores it

thereby protecting organs from ectopic deposition. Additionally, SAT has been positively associated with factors involved in energy intake regulation (leptin, adiponectin) and negatively associated with inflammatory factors (IL-6, TNFα, MCP-1) (Booth AD et al., 2018). The mechanisms underlying the fat redistribution are unclear yet because the effects of diet on metabolic and molecular consequences of regional adiposity have been scarcely studied. An association between diet quality and regional adiposity has been found in a study on large population of multi-ethnic Americans. Specifically, greater fruits, vegetables, whole grains, seeds/nuts and yogurt intake have been associated with decreased visceral adiposity, while red/processed meats have been associated with greater regional adiposity (Shah RV et al., 2016).

We verified also whether regular pistachio consumption modify somehow the intestinal microbiota of HFD mice because changes in the composition of the gut microbiota have been related to different metabolic disorders, including obesity (Cani et al., 2012; Álvarez-Mercado et al., 2019). Indeed, dysbiosis has been reported to increase intestinal permeability and gram-negative bacteria LPS in the circulation, leading to metabolic endotoxemia, adipose tissue dysfunction and systemic inflammation (Moreira et al., 2012; Cani et al., 2007).

Taken together, our results on the gut microbiota composition suggest a protective effect of chronic pistachio intake against dysbiosis. A number of different observations support our hypothesis:

1. The high Firmicutes to Bacteroidetes ratio (index of dysbiosis), found in HFD mice, was significantly lower in HFD-P group.
2. *Lactobacillus*, whose abundance is related to the reduction of endotoxemia, inflammation and improvement in the immune system (Hutchinson et al., 2020), was significantly increased in the HFD-P, in comparison to the other groups.
3. Pistachio diet significantly increased abundance of genera linked to a healthier profile such as *Parabacteroides*, *Dorea*, *Allobaculum*, *Turicibacter*, *Lactobacillus* and *Anaeroplasma*, while strongly inhibited the growth of bacteria associated with dysmetabolism and inflammation such as *Oscillospira*, *Desulfovibrio*, *Coprobacillus* and *Bilophila*.

However, our results do allow us to clarify if the dysbiosis improvement is the key mechanism by which regular pistachio intake ameliorates the systemic and consequently liver and adipose tissue inflammation. In order to conclude that the gut microbiota change is the main player responsible for the beneficial effects of pistachio intake on the obesity-related disorders we should examine the pistachio impact in absence of microbiota, using germ-free mice or animals treated with a cocktail of broad spectrum antibiotics.

Anyway, our Ussing chamber experiments highlighted that pistachios-based diet was able to prevent the increase in permeability of the intestinal barrier. This observation could support the hypothesis of the central role of gut microbiota in mediating pistachio intake benefits. The pistachio induced increase in *Lactobacillus* abundance observed in the present study could be at the origin of the improvement of gut barrier function, because a positive correlation between *Lactobacillus* abundance and intestinal trans-epithelial resistance has been reported (Lam et al. 2012). However, we cannot exclude a direct effect of pistachio or its individual components on epithelial cell barrier function. In support of this hypothesis, decrement of paracellular permeability induced by Sicilian pistachio hydrophilic extract in IL-1β-exposed human intestinal epithelial cells was reported (Gentile C et al., 2015).

Lastly, we took into account the possible impact of pistachio-based diet on the brain healthy conditions because it is now well accepted that obesity increase the risk of neurodegenerative disorders. (Keshk et al., 2020). Excessive free fatty acids can cross the BBB and enhance *de novo* synthesis of ceramide, which can induce brain IR as well as neuronal redox imbalance. Free fatty acids may increase the formation of intracellular ROS resulting in neuronal toxicity (Tan & Norhaizan, 2019). Moreover, lipids are target for free radicals and they play a crucial role in reactions that yield hydroperoxides, endoperoxides, and oxysterol, responsible for cellular damage.

Taken together, the results from the experiments in the brain of obese mice support the hypothesis that pistachio-supplemented diet can mitigate the harmful effects associated with the consumption of HFD. The evidences include:

1. Significantly higher levels of cholesterol and lipids were found in the HFD brain than HFD-P brain. As above mentioned, this property is probably due to the high content of mono- and polyunsaturated fatty acids and phytosterols,
2. The high levels of ROS and singlet oxygen in the brain of HFD mice were partially counteracted by the diet with pistachios.
3. Lipid peroxidation, reported to be increased in brains of AD patients (Chauhan & Chauhan, 2006) was significantly lower in HFD-P brain than HFD brain.
4. Expression of p-ERK, typical marker of oxidative stress, that was increased in HFD cerebral cortex sections, was decreased in HFD-P cortex.
5. Downregulation of SOD2 and H-Oxy, enzymatic antioxidant systems, present in the brain of HFD mice was prevented by HFD-P.
6. SOD2 activity was significantly higher in HFD-P compared to HFD.
7. HFD-induced neuronal apoptosis was reduced in HFD-P brains, as demonstrated by a reduced number of cells with fragmented DNA in the cortical areas.

8. Improvement of mitochondrial dysfunction was observed in HFD-P brain.

Indeed, although the underlying mechanisms are not completely understood, brain mitochondrial dysfunction is involved in the development of neurological and neurodegenerative diseases (Norat et al., 2020). Mitochondria specifically located at synapses may play a key role in providing energy to support synaptic functions and plasticity, thus their defects may lead to synaptic failure, which is a common hallmark of neurodegenerative diseases (Cavaliere et al., 2019). We found that HFD-P attenuated the high levels of oxidative species inside the mitochondria and it increased swelling. Moreover, HFD-P decreased the proteins involved in fission process, mitochondrial damage, mitophagy and lysosomal activity, in comparison with HFD mice.

Therefore, pistachio-based diet improves the endogenous antioxidant function associated with removing accumulated ROS, the balance between ROS and antioxidants, it reduces oxidative damage to lipids and thus, it improves the pathological characteristics of HFD-induced neurodegeneration. It should be interesting to evaluate the pistachio neuroprotective properties also in other models of neurodegeneration including motor function or memory impairments as well as to examine the possible link between effects on microbiota and neurodegeneration.

Overall, the results suggest that the integration of pistachios could be a safe nutritional strategy in order to prevent the risks associated with metabolic syndrome. Therefore, the beneficial properties of pistachio could justify possible use of pistachios on humans, as multi-target agent, easily incorporated into a healthy dietary pattern in the prevention of MetS.

References

Abraham TM, Pedley A, Massaro JM, Hoffmann U, Fox CS. Association between visceral and subcutaneous adipose depots and incident cardiovascular disease risk factors. *Circulation* 2015, 132, 1639–1647.

Adolph TE, Grander C, Grabherr F, Tilg H. Adipokines and Non-Alcoholic Fatty Liver Disease: Multiple Interactions. *Int. J. Mol. Sci*. 2017; 18:1649.

Ahmad NS, Waheed A, Farman M, Qayyum A. Analgesic and anti-inflammatory effects of pistacia integerrima extracts in mice. *J. Ethnopharmacol* 2010, 129, 250–253.

Aksoy N, Aksoy M, Bagci C, Gergerlioglu HS, Celik H, Herken E, Yaman A, Tarakcioglu M, Soydinc S, Sari I, Davutoglu V. Pistachio intake increases high density lipoprotein levels and inhibits low-density lipoprotein oxidation in rats. *Tohoku J Exp Med.* 2007, 212:43-48.

Alberti KG, Zimmet P, Shaw J. The metabolic syndrome—a new worldwide definition. IDF Epidemiology Task Force Consensus Group. *Lancet.* 2005; 366(9491):1059–1062.

Alberti KG, Zimmet PZ. Definition, diagnosis and classification of diabetes mellitus and its complications. Part1 diagnosis and classification of diabetes mellitus provisional report of a WHO consultation. *Diabet Med*. 1998;539-553.

Albuquerque D, Stice E, Rodríguez-López R, Manco L, Nóbrega C. Current review of genetics of human obesity: from molecular mechanisms to an evolutionary perspective. *Mol Genet Genomics*. 2015; 290(4):1191-221.

Aldemir M, Okulu E, Neşelioğlu S, Erel O, Kayıgil O. Pistachio diet improves erectile function parameters and serum lipid profiles in patients with erectile dysfunction. *Int J Impot Res*. 2011; 23:32-38.

Ali T, Badshah H, Kim T, Kim MO. Melatonin attenuates D-galactose-induced memory impairment, neuroinflammation and neurodegeneration via RAGE/NF-KB/ JNK signaling pathway in aging mouse model. *J Pineal Res*. 2015; 58, 71-85.

Alnahdi A, John A, Raza H. Augmentation of Glucotoxicity, Oxidative Stress, Apoptosis and Mitochondrial Dysfunction in HepG2 Cells by Palmitic Acid. *Nutrients.* 2019; 11(9): 1979.

Altmann SW, Davis HR, Jr Zhu LJ, Yao X, Hoos LM, Tetzloff G, Iyer SP, Maguire M, Golovko A, Zeng M, Wang L, Murgolo N, Graziano MP. Niemann-Pick C1 Like 1 protein is critical for intestinal cholesterol absorption. *Science*. 2004; 303:1201-1204.

Alturfan AA, Emekli-Alturfan E, Uslu E. Consumption of pistachio nuts beneficially affected blood lipids and total antioxidant activity in rats fed a high-cholesterol diet. *Folia Biol (Praha).* 2009, 55:132-136.

Álvarez-Mercado AI, Navarro-Oliveros M, Robles-Sánchez C, Plaza-Díaz J, Sáez-Lara MJ, Muñoz-Quezada S, Fontana L, Abadía-Molina F. Microbial population changes and their relationship with human health and disease. *Microorganisms.* 2019, 7, 68.

Amato A, Caldara GF, Nuzzo D, Baldassano S, Picone P, Rizzo M, Mulè F, Di Carlo M. NAFLD and Atherosclerosis Are Prevented by a Natural Dietary Supplement Containing Curcumin,

Silymarin, Guggul, Chlorogenic Acid and Inulin in Mice Fed a High-Fat Diet. *Nutrients.* 2017, 13;9(5).

Ammari M, Othman H, Hajri A, Sakly M, Abdelmelek H. Pistacia lentiscus oil attenuates memory dysfunction and decreases levels of biomarkers of oxidative stress induced by lipopolysaccharide in rats. *Brain Res Bull*. 2018; 140: 140-147.

Anderson JW, Hanna TJ, Peng X, Kryscio RJ. Whole grain foods and heart disease risk. *J Am Coll Nutr.* 2000; 19: 291S–299S.

Anhệ FF, Roy D, Pilon G, Dudonné S, Matamoros S, Varin TV, Garofalo C, Moine Q, Desjardins Y, Levy E, Marette A. A polyphenol-rich cranberry extract protects from diet-induced obesity, insulin resistance and intestinal inflammation in association with increased Akkermansia spp. Population in the gut microbiota of mice. *Gut* 2015, 64, 872–883.

Anjum I, Fayyaz M, Wajid A, Sohail W, Ali A. Does obesity increase the risk of dementia: a literature review. *Cureus.* 2018, 10(5):e2660.

Arab JP, Arrese M, Trauner M. Recent Insights into the Pathogenesis of Nonalcoholic Fatty Liver Disease. Annu. *Rev Pathol Mech Dis.* 2018; 13:321–350.

Arora T, Singh S, Sharma RK. Probiotics: Interaction with gut microbiome and antiobesity potential. *Nutrition*. 2013; 29:591–596.

Askari G, Yazdekhasti N, Mohammadifard N, Sarrafzadegan N, Bahonar A, Badiei M, Sajjadi F, Taheri M. The relationship between nut consumption and lipid profile among the Iranian adult population; Isfahan Healthy Heart Program. *Eur J Clin Nutr.* 2013, 67:385-9.

Assaf-Balut C, de la Torre N García, Durán A, Fuentes M, Bordiú E, del Valle L et al. A Mediterranean diet with additional extra virgin olive oil and pistachios reduces the incidence of gestational diabetes mellitus (GDM): A randomized controlled trial: The St. Carlos GDM prevention study. *PLoS One*. 2017; 12(10): e0185873.

Aubert H, Frere C, Aillaud MF, Morange PE, Juhan-Vague I, Alessi MC. Weak and non-independent association between plasma TAFI antigen levels and the insulin resistance syndrome. *J Thromb Haemost.* 2003;791-797.

Awień J, Nastałek P, Korbut R. Mouse models of experimental atherosclerosis. *J Physiol Pharmacol.* 2004; 55:503-517.

Ayoub HM, McDonald MR, Sullivan JA, Tsao R, Meckling KA. Proteomic Profiles of Adipose and Liver Tissues from an Animal Model of Metabolic Syndrome Fed Purple Vegetables. *Nutrients.* 2018; 10(4). pii: E456.

Bagheri S, Sarabi MM, Khosravi P, Khorramabadi RM, Veiskarami S, Ahmadvand H, Keshvari M. Effects of Pistacia atlantica on oxidative stress markers and antioxidant enzymes expression in diabetic rats. *J Am Coll Nutr.* 2019; 38, 267-274.

Balan KV, Prince J, Han Z, Dimas K, Cladaras M, Wyche JH, Sitaras NM, Pantazis P. Antiproliferative activity and induction of apoptosis in human colon cancer cells treated in vitro with constituents of a product derived from pistacia lentiscus l. Var. Chia. *Phytomedicine*. 2007, 14, 263-272.

Baldassano S, Amato A, Caldara GF, Mulè F. Glucagon-like peptide-2 treatment improves glucose dysmetabolism in mice fed a high-fat diet. *Endocrine.* 2016a; 54:648-656.

Baldassano S, Amato A, Cappello F, Rappa F, Mulè F. Glucagon-like peptide-2 and mouse intestinal adaptation to a high-fat diet. *J Endocrinol.* 2013; 217:11-20.

Baldassano S, Amato A, Rappa F, Cappello F, Mulè F. Influence of endogenous glucagon-like peptide-2 on lipid disorders in mice fed a high-fat diet. *Endocr Res*. 2016b; 41:317-324.

Baldassano S, Rappa F, Amato A, Cappello F, Mulè F. GLP-2 as Beneficial Factor in the Glucose Homeostasis in Mice Fed a High Fat Diet. *J Cell Physiol.* 2015; 230:3029-3036.

Balkau B, Charles MA. Comment on the provisional report from the WHO consultation. European Group for the Study of Insulin Resistance (EGIR). *Diabet Med.* 1999; 16(5):442-3.

Bastard JP, Jardel C, Bruckert E, Blondy P, Capeau J, Laville M, Vidal H, Hainque B. Elevated levels of interleukin 6 are reduced in serum and subcutaneous adipose tissue of obese women after weight loss. *J Clin Endocrinol Metab.* 2000; 85(9):3338-42.

Bays H. Central obesity as a clinical marker of adiposopathy; increased visceral adiposity as a surrogate marker for global fat dysfunction. *Curr Opin Endocrinol Diabetes Obes*. 2014; 21:345-351.

Beaumont M, Andriamihaja M, Lan A, Khodorova N, Audebert M, Blouin JM, Grauso M, Lancha L, Benetti PH, Benamouzig R, Tomé D, Bouillaud F, Davila AM, Blachier F. Detrimental effects for colonocytes of an increased exposure to luminal hydrogen sulfide: The adaptive response. *Free Radic Biol Med.* 2016; 93, 155-164.

Ben Khedir S, Mzid M, Bardaa S, Moalla D, Sahnoun Z, Rebai T. In vivo evaluation of the anti-inflammatory effect of pistacia lentiscus fruit oil and its effects on oxidative stress. *Evid Based Complement Alternat Med*. 2016; 2016:6108203.

Benedict M, Zhang X. Non-alcoholic fatty liver disease: An expanded review. *World J Hepatol.* 2017; 9(16): 715–732.

Bisignano C, Filocamo A, Faulks RM, Mandalari G. In vitro antimicrobial activity of pistachio (*Pistacia vera* L.) polyphenols. *FEMS Microbiol Lett*. 2013; 341, 62–67.

Boles A, Kandimalla R, Reddy PH. Dynamics of diabetes and obesity: Epidemiological perspective. Biochim *Biophys Acta Mol Basis Dis*. 2017; 1863(5):1026-1036.

Bongarzone S, Savickas V, Luzi F, Gee AD. Targeting the Receptor for Advanced Glycation Endproducts (RAGE): A Medicinal Chemistry Perspective. *J Med Chem*. 2017; 60(17): 7213–7232.

Bonomini F, Rodella LF, Rezzani R. Metabolic syndrome, aging and involvement of oxidative stress. *Aging Dis.* 2015; 6(2):109-20.

Bozzetto L, Costabile G, Della Pepa G, Ciciola P, Vetrani C, Vitale M, Rivellese AA, Annuzzi G. Dietary Fibre as a Unifying Remedy for the Whole Spectrum of Obesity-Associated Cardiovascular Risk. *Nutrients.* 2018; 10(7): 943.

Brown L, Poudyal H, Panchal SK. Functional foods as potential therapeutic options for metabolic syndrome. *Obes Rev*. 2015; 16, 914-941.

Byndloss MX, Olsan EE, Rivera-Chávez F, Tiffany CR, Cevallos SA, Lokken KL, Torres TP, Byndloss AJ, Faber F, Gao Y, Litvak Y, Lopez CA, Xu G, Napoli E, Giulivi C, Tsolis RM, Revzin A, Lebrilla CB, Bäumler AJ. Microbiota-activated PPAR-γ signaling inhibits dysbiotic Enterobacteriaceae expansion. *Science* 2017; 357, 570-575.

Cabral CE, Klein MRST. Phytosterols in the Treatment of Hypercholesterolemia and Prevention of Cardiovascular Diseases. *Arq Bras Cardiol*. 2017; 109: 475–482.

Caesar R, Tremaroli V, Kovatcheva-Datchary P, Cani PD, Bäckhed F. Crosstalk between Gut Microbiota and Dietary Lipids Aggravates WAT Inflammation through TLR Signaling. *Cell Metab.* 2015; 22: 658-668.

Călinoiu LF, Vodnar DC. Whole Grains and Phenolic Acids: A Review on Bioactivity, Functionality, Health Benefits and Bioavailability. *Nutrients*. 2018; 10(11): 1615.

Camps-Bossacoma M, Massot-Cladera M, Abril-Gil M, Franch A, Pérez-Cano FJ, Castell M. Cocoa Diet and Antibody Immune Response in Preclinical Studies. *Front Nutr.* 2017; 4, 28.

Cani PD, Amar J, Iglesias MA, Poggi M, Knauf C, Bastelica D, Neyrinck AM, Fava F, Tuohy KM, Chabo C, Waget A, Delmée E, Cousin B, Sulpice T, Chamontin B, Ferrières J, Tanti JF, Gibson GR, Casteilla L, Delzenne NM, Alessi MC, Burcelin R. Metabolic endotoxemia initiates obesity and insulin resistance. *Diabetes* 2007; 56: 1761-1772.

Cani PD, Bibiloni R, Knauf C, Waget A, Neyrinck AM, Delzenne NM, Burcelin R. Changes in gut microbiota control metabolic endotoxemia-induced inflammation in high-fat diet-induced obesity and diabetes in mice. *Diabetes* 2008; 57: 1470-1481.

Cani PD, Lecourt E, Dewulf EM, Sohet FM, Pachikian BD, Naslain D, De Backer F, Neyrinck AM, Delzenne NM. Gut microbiota fermentation of prebiotics increases satietogenic and incretin gut peptide production with consequences for appetite sensation and glucose response after a meal. *Am J Clin Nutr*. 2009; 90: 1236-1243.

Cani PD, Osto M, Geurts L, Everard A. Involvement of gut microbiota in the development of low-grade inflammation and type 2 diabetes associated with obesity. *Gut Microbes* 2012; 3: 279–288.

Carbajo-Pescador S, Porras D, García-Mediavilla MV, Martínez-Flórez S, Juarez-Fernández M, Cuevas MJ, Mauriz JL, González-Gallego J, Nistal E, Sánchez-Campos S. Beneficial effects of exercise on gut microbiota functionality and barrier integrity, and gut-liver crosstalk in an in vivo model of early obesity and non-alcoholic fatty liver disease. *Dis Model Mech.* 2019, 12, pii: dmm039206.

Carbone F, Mach F, Montecucco F. The role of adipocytokines in atherogenesis and atheroprogression. *Curr Drug Targets*. 2015; 16: 295-320.

Carughi A, Bellisle F, Dougkas A, Giboreau A, Feeney MJ, Higgs J. A Randomized Controlled Pilot Study to Assess Effects of a Daily Pistachio (Pistacia Vera) Afternoon Snack on Next-Meal Energy Intake, Satiety, and Anthropometry in French Women. *Nutrients.* 2019;11(4):767.

Carvalho JCT, Fernandes CP, Daleprane JB, Alves MS, Stien D Dhammika Nanayakkara NP. Role of Natural Antioxidants from Functional Foods in Neurodegenerative and Metabolic Disorders. *Oxid. Med. Cell. Longev*. 2018; 2018:1459753.

Casas R, Sacanella E, and Estruch R. The Immune Protective Effect of the Mediterranean Diet against Chronic Low-grade Inflammatory Diseases. *Endocr Metab Immune Disord Drug Targets.* 2016; 14(4): 245–254.

Castaner O, Goday A, Park YM, Lee SH, Magkos F, Toh Ee Shiow SA, Schröder H. The Gut Microbiome Profile in Obesity: A Systematic Review. *Int J Endocrinol*. 2018; 2018: 4095789.

Catalani S, Palma F, Battistelli S, Nuvoli B, Galati R, Benedetti S. Reduced cell viability and apoptosis induction in human thyroid carcinoma and mesothelioma cells exposed to cidofovir. *Toxicol. In Vitro* 2017; 41: 49-55.

Cavaliere G, Trinchese G, Penna E, Cimmino F, Pirozzi C, Lama A, Annunziata C, Catapano A, Mattace Raso G, Meli R, Monda M, Messina G, Zammit C, Crispino M, Mollica MP. High-Fat Diet Induces Neuroinflammation and Mitochondrial Impairment in Mice Cerebral Cortex and Synaptic Fraction. *Front Cell Neurosci*. 2019; 13: 509.

Chapa-Dubocq X, Makarov V, Javadov S. Simple kinetic model of mitochondrial swelling in cardiac cells. *J Cell Physiol.* 2018; 233: 5310-5321.

Charlton M, Sanyal AJ, Cusi K, Lavine JE, Brunt EM, Harrison SA, Younossi Z, Rinella M, Chalasani N, Younossi Z, et al. The diagnosis and management of nonalcoholic fatty liver disease: Practice guidance from the American Association for the Study of Liver Diseases. *Hepatology.* 2017; 67:328–357.

Charradi K, Mahmoudi M, Bedhiafi T, Kadri S, Elkahoui S, Limam F, Aouani E. Dietary supplementation of grape seed and skin flour mitigates brain oxidative damage induced by a high-fat diet in rat: Gender dependency. *Biomed. Pharmacother*. 2017; 87: 519-526.

Chaudhuri J, Bains Y, Guha S, Kahn A, Hall D, Bose N, Gugliucci A, Kapahi P. The Role of Advanced Glycation End Products in Aging and Metabolic Diseases: Bridging Association and Causality. *Cell Metab*. 2018; 28(3):337-352.

Chavez JA, Knotts TA, Wang LP, et al. A role for ceramide, but not diacylglycerol, in the antagonism of insulin signal transduction by saturated fatty acids. J Biol Chem. 2003; 278: 10297-10303.

Chen S, Chen Y, Ma S, Zheng R, Zhao P, Zhang L, Liu Y, Yu Q, Deng Q, Zhang K. Dietary fibre intake and risk of breast cancer: A systematic review and meta-analysis of epidemiological studies. *Oncotarget.* 2016; 7(49): 80980–80989.

Clemente-Postigo M, Oliva-Olivera W, Coin-Aragüez L, Ramos-Molina B, Giraldez-Perez RM, Lhamyani S, Alcaide-Torres J, Perez-Martinez P, El Bekay R, Cardona F, Tinahones FJ. Metabolic endotoxemia promotes adipose dysfunction and inflammation in human obesity. *Am J Physiol Metab.* 2019; 316: E319–E332.

Collins S, Martin TL, Surwit RS, Robidoux J. Genetic vulnerability to diet-induced obesity in the C57BL/6J mouse: physiological and molecular characteristics. *Physiol Behav* 2004; 81:243-248.

Crewe C, Zhu Y, Paschoal VA, Joffin N, Ghaben AL, Gordillo R, Oh DY, Liang G, Horton JD, Scherer PE. SREBP-regulated adipocyte lipogenesis is dependent on substrate availability and redox modulation of mTORC1. *JCI Insight.* 2019; 5: e129397.

D'Evoli L, Lucarini M, Gabrielli P, Aguzzi A, Lombardi-Boccia G. Nutritional value of italian pistachios from Bronte (*Pistacia vera*, L.) their nutrients, bioactive compounds and antioxidant activity. *Food and Nutrition Sciences.* 2015; 06: 10.4236/fns.2015.614132.

Dahl WJ, Stewart ML. Position of the Academy of Nutrition and Dietetics: Health Implications of Dietary Fiber. *J Acad Nutr Diet*. 2015;115(11):1861-70.

Dangles O, Fenger JA. The Chemical Reactivity of Anthocyanins and Its Consequences in Food Science and Nutrition. *Molecules.* 2018; 23(8): 1970.

David LA, Maurice CF, Carmody RN, Gootenberg DB, Button JE, Wolfe BE, Ling AV, Devlin AS, Varma Y, Fischbach MA, Biddinger SB, Dutton RJ, Turnbaugh PJ. Diet rapidly and reproducibly alters the human gut microbiome. Nature 2014; 505: 559-563.

De Filippo C, Cavalieri D, Di Paola M, Ramazzotti M, Poullet JB, Massart S, Collini S, Pieraccini G, Lionetti, P. Impact of diet in shaping gut microbiota revealed by a comparative study in children from Europe and rural Africa. *Proc Natl Acad Sci U S A* 2010; 107: 14691-14696.

De Souza RGM, Schincaglia RM, Pimente GD, Mota JF. Nuts and human health outcomes: a systematic review. *Nutrients.* 2017; 9: pii: E1311.

Del Gobbo LC, Falk MC, Feldman R, Lewis K, Mozaffarian D. Effects of tree nuts on blood lipids, apolipoproteins, and blood pressure: systematic review, meta-analysis, and dose-response of 61 controlled intervention trials. *Am J Clin Nutr*. 2015; 102:1347-56.

Delzenne NM, Neyrinck AM, Cani PD. Modulation of the gut microbiota by nutrients with prebiotic properties: consequences for host health in the context of obesity and metabolic syndrome. *Microb Cell Fact.* 2010; 10, Suppl 1: S10.

Desagher S, Martinou JC. Mitochondria as the central control point of apoptosis. *Trends Cell. Biol*. 2000; 10, 369–77.

Devkota S, Wang Y, Musch MW, Leone V, Fehlner-Peach H, Nadimpalli A, Antonopoulos DA, Jabri B, Chang EB. Dietary-fat-induced taurocholic acid promotes pathobiont expansion and colitis in Il10-/- mice. *Nature*. 2012; 487: 104-108.

Di Daniele N, Noce A, Vidiri MF, Moriconi E, Marrone G, Annicchiarico-Petruzzelli M, D'Urso G, Tesauro M, Rovella V, De Lorenzo A. Impact of Mediterranean diet on metabolic syndrome, cancer and longevity. *Oncotarget.* 2017; 8(5): 8947–8979.

Djelti F, Braudeau J, Hudry E, Dhenain M, Varin J, Bieche I, Marquer C, Chali F, Ayciriex S, Auzeil N, Alves S, Langui D, Potier MC, Laprevote O, Vidaud M, Duyckaerts C, Miles R, Aubourg P, Cartier N. CYP46A1 inhibition, brain cholesterol accumulation and neurodegeneration pave the way for Alzheimer's disease. *Brain.* 2015; 138: 2383-2398.

Donato AJ, Henson GD, Hart CR, Layec G, Trinity JD, Bramwell RC, Enz RA, Morgan RG, Reihl KD, Hazra S, Walker AE, Richardson RS, Lesniewski LA. The impact of ageing on adipose structure, function and vasculature in the B6D2F1 mouse: evidence of significant multisystem dysfunction. *J Physiol.* 2014; 592:4083-4096.

Dorothée G. Neuroinflammation in neurodegeneration: role in pathophysiology, therapeutic opportunities and clinical perspectives. *J Neural Transm*. 2018;125(5):749-750.

Dreher ML. Pistachio nuts: composition and potential health benefits. *Nutr Rev.* 2012; 70:234-40.

Dringen R. Glutathione metabolism and oxidative stress in neurodegeneration. *Eur J Biochem*. 2000; 267: 4903.

Eckel RH, Grundy SM, Zimmet PZ. The metabolic syndrome. *Lancet* 2005; 365: 1415-1428.

Eckel RH. Lipoprotein lipase. A multifunctional enzyme relevant to common metabolic diseases. *N Engl J Med.* 1989;1060-1068.

Edwards K, Kwaw I, Matud J, Kurtz I. Effect of pistachio nuts on serum lipid levels in patients with moderate hypercholesterolemia. *J Am Coll Nutr*. 1999; 18:229-232.

ElAli A, Doeppner TR, Zechariah A, Hermann DM. Increased blood-brain barrier permeability and brain edema after focal cerebral ischemia induced by hyperlipidemia: role of lipid peroxidation and calpain-1/2 matrix metalloproteinase-2/9, and RhoA overactivation. *Stroke.* 2011; 42: 3238–3244.

Elmaliklis IN, Liveri A, Ntelis B, Paraskeva K, Goulis I, Koutelidakis AE. Increased Functional Foods' Consumption and Mediterranean Diet Adherence May Have a Protective Effect in the Appearance of Gastrointestinal Diseases: A Case–Control Study. *Medicines (Basel*). 2019; 6(2):50.

Endo H, Niioka M, Kobayashi N, Tanaka M, Watanabe T. Butyrate-producing probiotics reduce nonalcoholic fatty liver disease progression in rats: new insight into the probiotics for the gut-liver axis. *PLoS One.* 2013; 8: e63388.

Esmat A, Al-**A**bbasi FA, Algandaby MM, Moussa AY, Labib RM, Ayoub NA, Abdel-Naim AB. Anti-inflammatory activity of pistacia khinjuk in different experimental models: Isolation and characterization of its flavonoids and galloylated sugars. *J Med Food.* 2012; 15: 278–287.

Evans J, Magee A, Dickman K, Sutter R, Sutter C. A Plant-Based Nutrition Program. *Am J Nurs*. 2017; 117: 56-61.

Fang F, Lue LF, Yan S, Xu H, Luddy JS, Chen D, Walker DG, Stern DM, Schmidt AM, Chen JX, Yan SS. RAGE-dependent signaling in microglia contributes to neuroinflammation, Abeta accumulation, and impaired learning/memory in a mouse model of Alzheimer's disease. *FASEB J.* 2010; 24:1043–1055.

Fantino M, Bichard C, Mistretta F, Bellisle F. Daily consumption of pistachios over 12 weeks improves dietary profile without increasing body weight in healthy women: A randomized controlled intervention. *Appetite.* 2020; 144: 104483.

Farag MA, Abdelwareth A, Sallam IE, el Shorbagi M, Jehmlich N, Fritz-Wallace K, Schäpe SS, Rolle-Kampczyk U, Ehrlich A, Wessjohann LA, von Bergen M. Metabolomics reveals impact of seven functional foods on metabolic pathways in a gut microbiota model. *J Adv Res*. 2020; 23: 47–59.

Folch J, Lees M, Sloane Stanley GH. A simple method for the isolation and purification of total lipides from animal tissues. *J Biol Chem.* 1957; 226:497-509.

Förstermann U, Xia N, Li H. Roles of Vascular Oxidative Stress and Nitric Oxide in the Pathogenesis of Atherosclerosis. *Circ Res.* 2017;120(4):713-735.

Fried SK, Bunkin DA, Greenberg AS. Omental and subcutaneous adipose tissues of obese subjects release interleukin-6: depot difference and regulation by glucocorticoid. *J Clin Endocrinol Metab*. 1998; 83(3):847-50.

Gebauer SK, West SG, Kay CD, Alaupovic P, Bagshaw D, Kris-Etherton PM. Effects of pistachios on cardiovascular disease risk factors and potential mechanisms of action: a dose-response study. *Am J Clin Nutr*. 2008; 88:651-659.

Gentile C, Allegra M, Angileri F, Pintaudi AM, Livrea MA, Tesoriere L. Polymeric proanthocyanidins from Sicilian pistachio (*Pistacia vera* L.) nut extract inhibit lipopolysaccharide-induced inflammatory response in RAW 264.7 cells. *Eur J Nutr.* 2012; 51:353–363.

Gentile C, Perrone A, Attanzio A, Tesoriere L, Livrea MA. Sicilian pistachio (*Pistacia vera* L.) nut inhibits expression and release of inflammatory mediators and reverts the increase of paracellular permeability in IL-1β-exposed human intestinal epithelial cells. *Eur J Nutr*. 2015; 54:811-821.

Gentile C, Tesoriere L, Butera D, Fazzari M, Monastero M, Allegra M, Livrea MA. Antioxidant activity of Sicilian pistachio (*Pistacia vera L*. var. Bronte) nut extract and its bioactive components. *J Agric Food Chem.* 2007; 55: 643-648.

Gentile CL, Weir TL. The gut microbiota at the intersection of diet and human health. *Science* 2018; 362: 776–780.

Ghorbani A, Varedi M, Hadjzadeh MA, Omrani GH. Type-1 diabetes induces depot-specific alterations in adipocyte diameter and mass of adipose tissues in the rat. *Exp Clin Endocrinol Diabetes.* 2010; 118:442-448.

Giordano A, Murano I, Mondini E, Perugini J, Smorlesi A, Severi I, Barazzoni R, Scherer PE, Cinti S. Obese adipocytes show ultrastructural features of stressed cells and die of pyroptosis. *J Lipid Res.* 2013; 54: 2423-2436.

Gonzalez-Castejon M, Rodriguez-Casado A. Dietary phytochemicals and their potential effects on obesity: A review. *Pharmacol Res.* 2011; 64:438–455.

Gorji N, Moeini R, Memariani Z. Almond, hazelnut and walnut, three nuts for neuroprotection in Alzheimer's disease: A neuropharmacological review of their bioactive constituents. *Pharmacol. Res*. 2018; 129:115-127.

Gregório BM, De Souza DB, de Morais Nascimento FA, Pereira LM, Fernandes-Santos C. The potential role of antioxidants in metabolic syndrome. *Curr Pharm Des*. 2016; 22(7):859-69.

Guarner F, Perdigon G, Corthier G, Salminen S, Koletzko B, Morelli L. Should yoghurt cultures be considered probiotic? *Br J Nutr*. 2005; 93: 783-786.

Guglielmotto M, Aragno M, Tamagno E, Vercellinatto I, Visentin S, Medana C, Catalano MG, Smith MA, Perry G, Danni O, Boccuzzi G, Tabaton M. AGEs/RAGE complex upregulates BACE1 via NF-kappaB pathway activation. *Neurobiol Aging*. 2012; 33:196, e113–e127.

Gulati S, Misra A, Pandey RM, Bhatt SP, Saluja S. Effects of pistachio nuts on body composition, metabolic, inflammatory and oxidative stress parameters in Asian Indians with metabolic syndrome: a 24-wk, randomized control trial. *Nutrition*. 2014; 30: 192–197.

Guo J, Han X, Zhan J, You Y, Huang W. Vanillin Alleviates High Fat Diet-Induced Obesity and Improves the Gut Microbiota Composition. *Front Microbiol.* 2018; 9:2733.

Gupta V, Mah XJ, Garcia MC, Antonypillai C, van der Poorten D. Oily fish, coffee and walnuts: Dietary treatment for nonalcoholic fatty liver disease. *World J Gastroenterol*. 2015; 21:10621-10635.

Hall JE, do Carmo JM, da Silva AA, Wang Z, Hall ME. Obesity-induced hypertension: interaction of neurohumoral and renal mechanisms. *Circ Res*. 2015; 116(6): 991–1006.

Hamer HM, Jonkers D, Venema K, Vanhoutvin S, Troost FJ, Brummer RJ. Review article: the role of butyrate on colonic function. *Aliment Pharmacol Ther.* 2008; 27: 104-119.

Han JM, Jo AN, Lee SM, Bae HS, Jun DW, Cho YK, Suk KT, Yoon JH, Ahn SB, Cho YJ, Kim SW, Jang EC. Associations between intakes of individual nutrients or whole food groups and non-alcoholic fatty liver disease among Korean adults. *J Gastroenterol Hepatol*. 2014; 29:1265-1272.

Hayden MS, West AP, Ghosh S. SnapShot: NF-kappaB signaling pathways. *Cell.* 2006; 127:1286–1287.

Haynes P, Liangpunsakul S, Chalasani N. Nonalcoholic fatty liver disease in individuals with severe obesity. *Clin Liver Dis.* 2004; 8: 535-547.

Her nández-AlonsoP, Salas-Salvadó J, Baldrich-Mora M, Mallol R, Correig X, Bulló M. Effect of pistachio consumption on plasma lipoprotein subclasses in pre-diabetic subjects. *Nutr Metab Cardiovasc Dis.* 2015; 25:396-402.

Herbello-Hermelo P, Lamas JP, Lores M, Domínguez-González R, Bermejo-Barrera P, Moreda-Piñeiro A. Polyphenol bioavailability in nuts and seeds by an in vitro dialyzability approach. *Food Chem.* 2018; 254:20-25.

Hercberg S, Kesse-Guyot E, Druesne-Pecollo N, et al. Incidence of cancers, ischemic cardiovascular diseases and mortality during 5-year follow-up after stopping antioxidant vitamins and minerals supplements: a postintervention follow-up in the SU.VI.MAX Study. *Int J Cancer*. 2010; 127: 1875–1881.

Hernández-Alonso P, Bulló M, Salas-Salvadó J. Pistachios for Health What Do We Know About This Multifaceted Nut? *Nutr Today*. 2016; 51(3): 133–138.

Hernández-Alonso P, Salas-Salvadó J, Baldrich-Mora M, Juanola-Falgarona M, Bulló M. Beneficial effect of pistachio consumption on glucose metabolism, insulin resistance, inflammation, and related metabolic risk markers: a randomized clinical trial. *Diabetes Care.* 2014; 37(11):3098-105.

Hijmans BS, Grefhorst A, Oosterveer MH, Groen AK. Zonation of glucose and fatty acid metabolism in the liver: Mechanism and metabolic consequences. *Biochimie.* 2014; 96:121–129.

Ho HJ, Komai M, Shirakawa H. Beneficial Effects of Vitamin K Status on Glycemic Regulation and Diabetes Mellitus: A Mini-Review. *Nutrients.* 2020; 12(8): 2485.

Holligan SD, West SG, Gebauer SK, Kay CD, Kris-Etherton PM. A moderate-fat diet containing pistachios improves emerging markers of cardiometabolic syndrome in healthy adults with elevated LDL levels. *Br J Nutr*. 2014; 112:744-752.

Hong H, Dela Cruz JF, Kim WS, Yoo K, Hwang SG. Glehnia littoralis Root Extract Inhibits Fat Accumulation in 3T3-L1 Cells and High-Fat Diet-Induced Obese Mice by Downregulating Adipogenic Gene Expression. *Evid Based Complement Alternat Med*. 2018a; 2018:1243049.

Hong MY, Groven S, Marx A, Rasmussen C, Beidler J. Anti-Inflammatory, Antioxidant, and Hypolipidemic Effects of Mixed Nuts in Atherogenic Diet-Fed Rats. *Molecules*. 2018b; 23: pii: E3126.

Hopkins M, Blundell JE. Energy balance, body composition, sedentariness and appetite regulation: pathways to obesity. *Clin Sci (Lond*). 2016; 130(18):1615-28.

Hotamisligil GS, Murray DL, Choy LN, Spiegelman BM. Tumor necrosis factor alpha inhibits signaling from the insulin receptor. *Proc Natl Acad Sci USA*. 1994; 91(11):4854-8.

Hruby A, Hu FB. The Epidemiology of Obesity: A Big Picture. *Pharmacoeconomics* 2015; 33: 673-89.

Huang JH, Zhang C, Zhang DG, Li L, Chen X, Xu DX. Rifampicin-Induced Hepatic Lipid Accumulation: Association with Up-Regulation of Peroxisome Proliferator-Activated Receptor γ in Mouse Liver. *PLoS One*. 2016; 11: e0165787.

Innes JK, Calder PC. The Differential Effects of Eicosapentaenoic Acid and Docosahexaenoic Acid on Cardiometabolic Risk Factors: A Systematic Review. *Int J Mol Sci*. 2018; 19(2): 532.

Jakobsson HE, Rodríguez-Piñeiro AM, Schütte A, Ermund A, Boysen P, Bemark M, Sommer F, Bäckhed F, Hansson GC, Johansson ME. The composition of the gut microbiota shapes the colon mucus barrier. *EMBO Rep*. 2015; 16: 164-177.

Jamshidi S, Hejazi N, Golmakani MT, and Tanideh N. Wild pistachio (Pistacia atlantica mutica) oil improve metabolic syndrome features in rats with high fructose ingestion. *Iran J Basic Med Sci*. 2018; 21(12): 1255–1261.

Jenkins DJ, Kendall CW, Banach MS, Srichaikul K, Vidgen E, Mitchell S, Parker T, Nishi S, Bashyam B, de Souza R, Ireland C, Josse RG. Nuts as a replacement for carbohydrates in the diabetic diet. *Diabetes Care.* 2011; 34: 1706-1711.

Jensen MD, Caruso M, Heiling V, Miles JM. Insulin regulation of lipolysis in nondiabetic and IDDM subjects. *Diabetes.* 1989; 38: 1595-1601.

Jia Z, Pei Z, Maiguel D, Toomer CJ, Watkins PA. The fatty acid transport protein (FATP) family: very long chain acyl-CoA synthetases or solute carriers? *J Mol Neurosci.* 2007; 33:25-31.

Jiao N, Baker SS, Nugent CA, Tsompana M, Cai L, Wang Y, Buck MJ, Genco RJ, Baker RD, Zhu R, Zhu L. Gut microbiome may contribute to insulin resistance and systemic inflammation in obese rodents: a meta-analysis. *Physiol Genomics* 2018; 50: 244-254.

Johnson AM, Costanzo A, Gareau MG, Armando AM, Quehenberger O, Jameson JM, Olefsky JM. High fat diet causes depletion of intestinal eosinophils associated with intestinal permeability. *PLoS One* 2015; 10: e0122195.

Jump DB. Fatty acid regulation of hepatic lipid metabolism. *Curr Opin Clin Nutr Metab Care*. 2011; 14:115-120.

Kaushal V, Dye R, Pakavathkumar P, Foveau B, Flores J, Hyman B, Ghetti B, Koller BH, LeBlanc AC. Neuronal NLRP1 inflammasome activation of Caspase-1 coordinately regulates inflammatory interleukin-1-beta production and axonal degeneration-associated Caspase-6 activation. *Cell Death Differ*. 2015; 22(10):1676-1686.

Kelley DE, He J, Menshikova EV, Ritov VB. Dysfunction of mitochondria in human skeletal muscle in type 2 diabetes. *Diabetes.* 2002; 51: 2944-2950.

Kendall CW, West SG, Augustin LS, Esfahani A, Vidgen E, Bashyam B, Sauder KA, Campbell J, Chiavaroli L, Jenkins AL, Jenkins DJ. Acute effects of pistachio consumption on glucose and insulin, satiety hormones and endothelial function in the metabolic syndrome. *Eur J Clin Nutr*. 2014; 68:370-375.

Keshk WA, Ibrahim MA, Shalaby SM, Zalat ZA, Elseady WS. Redox status, inflammation, necroptosis and inflammasome as indispensable contributors to high fat diet (HFD)-induced neurodegeneration; Effect of N-acetylcysteine (NAC). *Arch Biochem Biophys*. 2020; 680: 108227.

Khedir SB, Mzid M, Bardaa S, Moalla D, Sahnoun Z, Rebai T. In Vivo Evaluation of the Anti-Inflammatory Effect of Pistacia lentiscus Fruit Oil and Its Effects on Oxidative Stress. *Evid Based Complement Alternat Med.* 2016; 2016: 6108203.

Kim HJ, Neophytou C. Natural anti-inflammatory compounds for the management and adjuvant therapy of inflammatory bowel disease and its drug delivery system. *Arch Pharm Res*. 2009; 32: 997–1004.

Kim SH, Park HS, Hong MJ, Hur HJ, Kwon DY, Kim MS. Caffeic Acid Phenethyl Ester Improves Metabolic Syndrome by Activating PPAR-γ and Inducing Adipose Tissue Remodeling in Diet-Induced Obese Mice. *Mol Nutr Food Res*. 2018; 62: e1700701.

Kim YB, Shulman GI, Kahn BB. Fatty acid infusion selectively impairs insulin action on Akt1 and protein kinase C lambda/zeta but not on glycogen synthase kinase-3. *J Biol Chem*. 2002; 277: 32915-32922.

Kleiner DE, Brunt EM. Nonalcoholic fatty liver disease: pathologic patterns and biopsy evaluation in clinical research. *Semin Liver Dis*. 2012; 32: 3-13.

Kocyigit A, Koylu AA, Keles H. Effects of pistachio nuts consumption on plasma lipid profile and oxidative status in healthy volunteers. *Nutr Metab Cardiovasc Dis*. 2006; 16:202-209.

Kritis AA, Stamoula EG, Paniskaki KA, Vavillis TD. Researching glutamate –induced cytotoxicity in differentcell lines: A comparative/collective analysis /study. *Front. Cell. Neurosci*. 2015; 9: 91.

Krstic D, Madhusudan A, Doehner J, Vogel P, Notter T, Imhof C, Manalastas A, Hilfiker M, Pfister S, Schwerdel C, Riether C, Meyer U, Knuesel I. Systemic immune challenges trigger and drive Alzheimer-like neuropathology in mice. *J Neuroinflammation.* 2012; 9:151.

Kylin E. Studies of the hypertension hyperglycemia hyperuricemia syndrome. *Zentralbl Innere Med*. 1923; 44: 105–27.

Lackey DE, Olefsky JM. Regulation of metabolism by the innate immune system. *Nat Rev Endocrinol* 2015; 12: 15–28.

Lai CS, Liao SN, Tsai ML, Kalyanam N, Majeed M, Majeed A, Ho CT, Pan MH. Calebin-A inhibits adipogenesis and hepatic steatosis in high-fat diet-induced obesity via activation of AMPK signaling. *Mol Nutr Food Res*. 2015; 59:1883-1895.

Lee SJ, Han JM, Lee JS, Son CG, Im HJ, Jo HK, Yoo HR, Kim YS, Seol IC. ACE Reduces Metabolic Abnormalities in a High-Fat Diet Mouse Model. *Evid Based Complement Alternat Med*. 2015; 2015:352647.

Léveillé M, Estall JL. Mitochondrial Dysfunction in the Transition from NASH to HCC. *Metabolites.* 2019; 9(10): 233.

Ley RE, Bäckhed F, Turnbaugh P, Lozupone CA, Knight RD, Gordon JI. Obesity alters gut microbial ecology. *Proc Natl Acad Sci USA*. 2005; 102: 11070-11075.

Li J, Tang Y, Cai D. IKKbeta/NF-kappaB disrupts adult hypothalamic neural stem cells to mediate a neurodegenerative mechanism of dietary obesity and pre-diabetes. *Nat Cell Biol*. 2012; 14:999–1012.

Li L, Acioglu C, Heary RF, Elkabesa Stella. Role of astroglial toll-like receptors (TLRs) in central nervous system infections, injury and neurodegenerative diseases. *Brain Behav Immun*. 2020; 91:740-755.

Li X, Wang H, Wang T, Zheng F, Wang H, Wang C. Dietary wood pulp-derived sterols modulation of cholesterol metabolism and gut microbiota in high-fat-diet-fed hamsters. *Food Funct.* 2019; 10: 775-785.

Liang W, Menke AL, Driessen A, Koek GH, Lindeman JH, Stoop R, Havekes LM, Kleemann R, van den Hoek AM. Establishment of a general NAFLD scoring system for rodent models and comparison to human liver pathology. *PLoS One.* 2014; 9: e115922.

Liang Y, Zhang Y, Deng Y, Liang S, He Y, Chen Y, Liu C, Lin C, Han L, Tu G, Yang Q. Chaihu-Shugan-San Decoction Modulates Intestinal Microbe Dysbiosis and Alleviates Chronic Metabolic Inflammation in NAFLD Rats via the NLRP3 Inflammasome Pathway. *Evid Based Complement Alternat Med.* 2018; 2018:9390786.

Liberale L, Bonaventura A, Vecchie A, Matteo C, Dallegri F, Montecucco F, et al. The role of adipocytokines in coronary atherosclerosis. *Curr Atheroscler Rep*. 2017;19: 10.

Lin MT, Beal MF. Mitochondrial dysfunction and oxidative stress in neurodegenerative diseases. *Nature.* 2006; 443: 787–795.

Liu JP, Tang Y, Zhou S, Toh BH, McLean C, Li H. Cholesterol involvement in the pathogenesis of neurodegenerative diseases. *Mol Cell Neurosci*. 2010; 43: 33-42.

Liu W, Crott JW, Lyu L, Pfalzer AC, Li J, Choi SW, Yang Y, Mason JB, Liu Z. Diet- and Genetically-induced Obesity Produces Alterations in the Microbiome, Inflammation and Wnt Pathway in the Intestine of Apc+/1638N Mice: Comparisons and Contrasts. *J Cancer* 2016; 7: 1780-1790.

Lonardo A, Nascimbeni F, Maurantonio M, Marrazzo A, Rinaldi L, Adinolfi LE. Nonalcoholic fatty liver disease: Evolving paradigms. *World J Gastroenterol*. 2017; 23(36):6571-6592.

Lopes-Vicente WRP, Rodrigues S, Cepeda FX, Jordão CP, Costa-Hong V, Dutra-Marques ACB, Carvalho JC, Alves MJNN, Bortolotto LA, Trombetta IC. Arterial stiffness and its association with clustering of metabolic syndrome risk factors. *Diabetol Metab Syndr*. 2017; 9: 87.

Lue LF, Walker DG, Brachova L, Beach TG, Rogers J, Schmidt AM, Stern DM, Yan SD. Involvement of microglial receptor for advanced glycation endproducts (RAGE) in Alzheimer's disease: identification of a cellular activation mechanism. *Exp Neurol*. 2001; 171:29–45.

Lumeng CN, Saltiel AR. Inflammatory links between obesity and metabolic disease. *J Clin Invest*. 2011; 121: 2111-2017.

Lyn-Cook LE Jr., Lawton M, Tong M, Silbermann E, Longato L, Jiao P, Mark P, Wands JR, Xu H, de la Monte SM. Hepatic ceramide may mediate brain insulin resistance and neurodegeneration in type 2 diabetes and non-alcoholic steatohepatitis. *J. Alzheimers Dis*. 2009; 16: 715-729.

Ma LY, Fei YL, Wang XY, Wu SD, Du JH, Zhu M, Jin L, Li M, Li HL, Zhai JJ, Ji LP, Ma RR, Liu SF, Li M, Ma L, Ma Xr, Qu QM, Lv YL. The Research on the Relationship of RAGE, LRP-1, and Aβ Accumulation in the Hippocampus, Prefrontal Lobe, and Amygdala of STZ-Induced Diabetic Rats. *J Mol Neurosci*. 2017; 62(1):1-10.

Makki K, Froguel P, Wolowczuk I. Adipose tissue in obesity-related inflammation and insulin resistance: cells, cytokines, and chemokines. *ISRN Inflamm*. 2013; e139239.

Mandalari G, Bisignano C, Filocamo A, Chessa S, Sarò M, Torre G, Faulks RM, Dugo P. Bioaccessibility of pistachio polyphenols, xanthophylls, and tocopherols during simulated human digestion. *Nutrition.* 2013; 29: 338-344.

Mankowski RT, Anton SD, Buford TW, Leeuwenburgh C. Dietary Antioxidants as Modifiers of Physiologic Adaptations to Exercise. *Med Sci Sports Exerc*. 2015; 47: 1857–1868.

Marchesi JR, Adams DH, Fava F, Hermes GD, Hirschfield GM, Hold G, Quraishi MN, Kinross J, Smidt H, Tuohy KM, Thomas LV, Zoetendal EG, Hart A. The gut microbiota and host health: a new clinical frontier. *Gut* 2016; 65: 330-339.

Marinou KA, Georgopoulou K, Agrogiannis G, Karatzas T, Iliopoulos D, Papalois A et al. Differential effect of Pistacia vera extracts on experimental atherosclerosis in the rabbit animal model: an experimental study. *Lipids Health Dis*. 2010; 9: 73.

Martinez-Gonzalez MA, Salas-Salvado J, Estruch R, et al. Benefits of the Mediterranean diet: insights from the PREDIMED Study. *Prog Cardiovasc Dis*. 2015; 58:50–60.

McCrickerd K, Forde CG. Sensory influences on food intake control: moving beyond palatability. *Obes Rev*. 2016;17(1):18-29.

McCullough A, Previs S, Kasumov T. Stable isotope-based flux studies in nonalcoholic fatty liver disease. *Pharmacol Ther*. 2018; 181: 22–33.

Mehla K, Balwani S, Kulshreshtha A, Nandi D, Jaisankar P Ghosh B. Ethyl gallate isolated from pistacia integerrima linn. Inhibits cell adhesion molecules by blocking ap-1 transcription factor. *J Ethnopharmacol*. 2011; 137: 1345–1352.

Mehta A, Prabhakar M, Kumar P, Deshmukh R, Sharma PL. Excitotoxicity: Bridge to various triggers in neurodegenerative disorders. *Eur J Pharmacol*. 2013; 698: 6-18.

Merone L, McDermott R. Nutritional anti-inflammatories in the treatment and prevention of type 2 diabetes mellitus and the metabolic syndrome. *Diabetes Res Clin Pract*. 2017; 127:238-253.

Moreira APB, Texeira TFS, Ferreira AB, do Carmo Gouveia Peluzio M, de Cássia Gonçalves Alfenas R. Influence of a high-fat diet on gut microbiota, intestinal permeability and metabolic endotoxaemia. *Br J Nutr* 2012; 108: 801–809.

Mori T, Paris D, Town T, Rojiani AM, Sparks DL, Delledonne A, Crawford F, Abdullah LI, Humphrey JA, Dickson DW, Mullan MJ. Cholesterol accumulates in senile plaques of Alzheimer disease patients and in transgenic APP(sw) mice. *J Neuropathol Exp Neurol*. 2001; 60: 778-785.

Mozaffarian D, Ludwig DS. The 2015 US Dietary Guidelines: lifting the ban on total dietary fat. *JAMA*. 2015; 313:2421–2422.

Myles IA. Fast food fever: Reviewing the impacts of the Western diet on immunity. *Nutr J.* 2014; 13: 61.

Nagalingam NA, Kao JY, Young VB. Microbial ecology of the murine gut associated with the development of dextran sodium sulfate-induced colitis. *Inflamm Bowel Dis*. 2011; 17: 917-926.

Naoi M, Wu Y, Shamoto-Nagai M, Maruyama W. Mitochondria in Neuroprotection by Phytochemicals: Bioactive Polyphenols Modulate Mitochondrial Apoptosis System, Function and Structure. *Int J Mol Sci.* 2019; 20: 2451.

Naouar MS, Mekki LZ, Charfi L, Boubaker J, Filali A. Preventive and curative effect of pistacia lentiscus oil in experimental colitis. *Biomed Pharmacother*. 2016; 83: 577–583.

Nguyen P, Leray V, Diez M, Serisier S, Le Bloc'h J, Siliart B, Dumon H. Liver lipid metabolism. *J Anim Physiol Anim Nutr (Berl)*. 2008; 92:272-83.

Nishida K, Otsu K. Inflammation and metabolic cardiomyopathy. *Cardiovasc Res.* 2017; 113(4):389-398.

Norat P, Soldozy S, Sokolowski JD, Gorick CM, Kumar JS, Chae Y et al. Mitochondrial dysfunction in neurological disorders: Exploring mitochondrial transplantation. *NPJ Regen Med*. 2020; 5: 22.

Nuzzo D, Amato A, Picone P, Terzo S, Galizzi G, Bonina FP, Mulè F, Di Carlo M. A natural dietary supplement with a combination of nutrients prevents neurodegeneration Induced by a high fat diet in mice. *Nutrients.* 2018; 10: pii: E1130.

Nuzzo D, Baldassano S, Amato A, Picone P, Galizzi G, Caldara GF, Di Carlo M, Mulè F. Glucagon-like peptide-2 reduces the obesity-associated inflammation in the brain. *Neurobiol Dis.* 2019; 121: 296-304.

Nuzzo D, Contardi M, Kossyvaki D, Picone P, Cristaldi L, Galizzi G, Bosco G, Scoglio S, Athanassiou A, Di Carlo M. Heat-resistant Aphanizomenon flos-aquae (AFA) extract (Klamin®) as a functional ingredient in food strategy for prevention of oxidative stress. *Oxid Med Cell Longev*. 2019; 2019:9481390.

Nuzzo D, Galizzi G, Amato A, Terzo S, Picone P, Cristaldi L, Mulè F, Di Carlo M. Regular Intake of Pistachio Mitigates the Deleterious Effects of a High Fat-Diet in the Brain of Obese Mice. *Antioxidants (Basel).* 2020; 9(4):317.

Nuzzo D, Picone P, Baldassano S, Caruana L, Messina E, Marino Gammazza A, Cappello F, Mulè F, Di Carlo M. Insulin resistance as common molecular denominator linking obesity to Alzheimer's disease. Curr Alzheimer Res. 2015; 12:723-735.

Oda E. Metabolic syndrome: its history, mechanisms, and limitations. *Acta Diabetol*. 2012; 49(2):89-95.

Olefsky JM, Glass CK. Macrophages, inflammation, and insulin resistance. *Annu Rev Physiol*. 2010; 72, 219-246.

Oliveira Andrade JM, Paraíso AF, Garcia ZM, Ferreira AV, Sinisterra RD, Sousa FB, Guimarães AL, de Paula AM, Campagnole-Santos MJ, dos Santos RA Santos SH. Cross talk between angiotensin-(1-7)/Mas axis and sirtuins in adipose tissue and metabolism of high-fat feed mice. *Peptides.* 2014; 55:158-65.

Oppi S, Lüscher TF, Stein Sokrates. Mouse Models for Atherosclerosis Research—Which Is My Line? *Front Cardiovasc Med.* 2019; 6: 46.

Panche AN, Diwan AD, Chandra SR. Flavonoids: an overview. *J Nutr Sci.* 2016; 5: e47.

Papada E, Forbes A, Amerikanou C, Torović L, Kalogeropoulos N, Tzavara C. Antioxidative Efficacy of a Pistacia Lentiscus Supplement and Its Effect on the Plasma Amino Acid Profile in Inflammatory Bowel Disease: A Randomised, Double-Blind, Placebo-Controlled Trial. *Nutrients.* 2018; 10(11): 1779.

Papalois A, Gioxari A, Kaliora AC, Lymperopoulou A, Agrogiannis G, Papada E, Andrikopoulos NK. Chios mastic fractions in experimental colitis: Implication of the nuclear factor kappab pathway in cultured ht29 cells. *J Med Food* 2012; 15: 974-983.

Parham M, Heidari S, Khorramirad A, Hozoori M, Hosseinzadeh F, Bakhtyari L, Vafaeimanesh J. Effects of Pistachio Nut Supplementation on Blood Glucose in Patients with Type 2 Diabetes: A Randomized Crossover Trial. *Rev Diabet Stud*. 2014; 11(2): 190–196.

Park YW, Zhu S, Palaniappan L, Heshka S, Carnethon MR, Heymsfield SB. The metabolic syndrome: prevalence and associated risk factor findings in the US population from the Third National Health and Nutrition Examination Survey, 1988-1994. *Arch Intern Med*. 2003;163(4):427-36.

Paterniti I, Impellizzeri D, Cordaro M, Siracusa R, Bisignano C, Gugliandolo E. The Anti-Inflammatory and Antioxidant Potential of Pistachios (*Pistacia vera* L.) *In Vitro and In Vivo*. *Nutrients.* 2017; 9(8): 915.

Pem D, Jeewon R. Fruit and Vegetable Intake: Benefits and Progress of Nutrition Education Interventions- Narrative Review Article. *Iran J Public Health*. 2015; 44(10): 1309–1321.

Perdomo L, Beneit N, Otero YF, Escribano Ó, Díaz-Castroverde S, Gómez-Hernández A, Benito M. Protective role of oleic acid against cardiovascular insulin resistance and in the early and late cellular atherosclerotic process. *Cardiovasc Diabetol*. 2015; 14:75.

Petersen KF, Befroy D, Dufour S, Dziura J, Ariyan C, Rothman DL, Di Pietro L, Cline GW, Shulman GI. Mitochondrial dysfunction in the elderly: possible role in insulin resistance. *Science.* 2003; 300:1140-1142.

Petersen KF, Dufour S, Befroy D, Garcia R, Shulman GI. Impaired mitochondrial activity in the insulin-resistant offspring of patients with type 2 diabetes. *N Engl J Med.* 2004; 350: 664-671.

Phillips CM. Nutrigenetics and Metabolic Disease: Current Status and implications for Personalised Nutrition. *Nutrients*. 2013; 5, 32-57.

Picone P, Giacomazza D, Vetri V, Carrotta R, Militello V, San Biagio PL, Di Carlo M. Insulin activated Akt rescues Ab oxidative stress induced cell death by orchestrating molecules trafficking. *Aging Cell*. 2011; 10:832-43.

Piya MK, Harte AL, McTernan PG. Metabolic endotoxaemia: is it more than just a gut feeling? *Curr Opin Lipidol*. 2013; 24:78-85.

Purkayastha S, Zhang G, Cai D. Uncoupling the mechanisms of obesity and hypertension by targeting hypothalamic IKK-beta and NF-kappaB. *Nat Med.* 2011; 17:883–887.

Qi L, Cho YA. Gene-environment interaction and obesity. *Nutr Rev*. 2008; 66(12): 684–694.

Qiao J, Li A, Jin X, Wang J. Mastic alleviates allergic inflammation in asthmatic model mice by inhibiting recruitment of eosinophils. *Am J Respi Cell Mol Biol*. 2011, 45, 95-100.

Ribeiro PVM, Silva A, Almeida AP, Hermsdorff HH, Alfenas RC. Effect of chronic consumption of pistachios (Pistacia vera L.) on glucose metabolism in pre-diabetics and type 2 diabetics: A systematic review. *Crit Rev Food Sci Nutr*. 2019; 59: 1115-1123.

Ridaura VK, Faith JJ, Rey FE, Cheng J, Duncan AE, Kau AL, Griffin NW, Lombard V, Henrissat B, Bain JR, Muehlbauer MJ, Ilkayeva O, Semenkovich CF, Funai K, Hayashi DK, Lyle BJ, Martini MC, Ursell LK, Clemente JC, Van Treuren W, Walters WA, Knight R, Newgard CB, Heath AC, Gordon JI. Gut microbiota from twins discordant for obesity modulate metabolism in mice. *Science* 2013; 341: 1241214.

Ritze Y, Bárdos G, Claus A, Ehrmann V, Bergheim I, Schwiertz A, Bischoff SC. Lactobacillus rhamnosus GG protects against non-alcoholic fatty liver disease in mice. *PLoS One* 2014; 9: e80169.

Rizzo NS, Sabaté J, Jaceldo-Siegl K, Fraser GE. Vegetarian dietary patterns are associated with a lower risk of metabolic syndrome: The adventist health study 2. *Diabetes Care* 2011; 3: 1225-1227.

Robbins GR, Wen H, Ting JP. Inflammasomes and metabolic disorders: old genes in modern diseases. *Mol Cell*. 2014; 54(2):297-308.

Rock CL, Zunshine E, Nguyen HT, Perez AO, Zoumas C, Pakiz B, White MM. Effects of Pistachio Consumption in a Behavioral Weight Loss Intervention on Weight Change, Cardiometabolic Factors, and Dietary Intake. *Nutrients.* 2020; 12(7):2155.

Rodríguez-Bencomo JJ, Kelebek H, Sonmezdag AS, Rodríguez-Alcalá LM, Fontecha J, Selli S. Characterization of the Aroma-Active, Phenolic, and Lipid Profiles of the Pistachio (Pistacia vera L.) Nut as Affected by the Single and Double Roasting Process. *J Agric Food Chem.* 2015; 63(35):7830-9.

Rojas-Gutierrez E, Muñoz-Arenas G, Treviño S, Espinosa B, Chavez R, Rojas K, Flores G, Díaz A, Guevara J. Alzheimer's disease and metabolic syndrome: A link from oxidative stress and inflammation to neurodegeneration. *Synapse.* 2017; 71: e21990.

Roopchand DE, Carmody RN, Kuhn P, Moskal K et al. Dietary polyphenols promote growth of the gut bacterium akkermansia muciniphila and attenuate high-fat diet induced metabolic syndrome. *Diabetes* 2015; 64: 2847–2858.

Ros E. Health Benefits of Nut Consumption. *Nutrients.* 2010; 2: 652–682.

Rossmeis M, Rim JS, Koza RA, Kozak LP. Variation in type 2 diabetes–related traits in mouse strains susceptible to diet-induced obesity. *Diabetes* 2003; 52: 1958–1966.

Roy B, Ehlert L, Mullur R, Freeby MJ, Woo MA, Kumar R, Choi S. Regional Brain Gray Matter Changes in Patients with Type 2 Diabetes Mellitus. *Sci Rep*. 2020;10(1):9925.

Ruegsegger GN, Vanderboom PM, Dasari S, Klaus KA, Kabiraj P, McCarthy CB, Lucchinetti CF, Nair KS. Exercise and metformin counteract altered mitochondrial function in the insulin-resistant brain. *JCI Insight*. 2019; 4: pii: 130681.

Rusinek H, Convit A. Obesity: cerebral damage in obesity-associated metabolic syndrome. *Nat Rev Endocrinol.* 2014; 10: 642-4.

Sabatè J, Ang Y. Nuts and health outcomes: new epidemiologic evidence. *Am J Clin Nutr.* 2009; 89: 1643S-1648S.

Saito M, Matsushita M, Yoneshiro T, Okamatsu-Ogura Y. Brown Adipose Tissue, Diet-Induced Thermogenesis, and Thermogenic Food Ingredients: From Mice to Men. *Front Endocrinol (Lausanne)*. 2020; 11: 222.

Salas-Salvadó J, Guasch-Ferré M, Lee CH, Estruch R, Clish CB, and Ros E. Protective Effects of the Mediterranean Diet on Type 2 Diabetes and Metabolic Syndrome. *J Nutr*. 2016; 146(4): 920S–927S.

Samuel VT, Liu ZX, Qu X, et al. Mechanism of hepatic insulin resistance in non-alcoholic fatty liver disease. *J Biol Chem*. 2004; 279:32345-32353.

Sa-Nguanmoo P, Tanajak P, Kerdphoo S, Jaiwongkam T, Pratchayasakul W, Chattipakorn N, Chattipakorn SC. SGLT2-inhibitor and DPP-4 inhibitor improve brain function via attenuating mitochondrial dysfunction, insulin resistance, inflammation, and apoptosis in HFD-induced obese rats. *Toxicol Appl Pharmacol*. 2017; 333: 43-50.

Santisteban MM, Qi Y, Zubcevic J, Kim S, Yang T, Shenoy V, Cole-Jeffrey CT, Lobaton GO, Stewart DC, Rubiano A, Simmons CS, Garcia-Pereira F, Johnson RD, Pepine CJ, Raizada MK. Hypertension-Linked Pathophysiological Alterations in the Gut. *Circ Res.* 2017; 120: 312-323.

Sari I, Baltaci Y, Bagci C, Davutoglu V, Erel O, Celik H, Ozer O, Aksoy N, Aksoy M. Effect of pistachio diet on lipid parameters, endothelial function, inflammation, and oxidative status: a prospective study. *Nutrition.* 2010; 26(4):399-404.

Sasaki N, Toki S, Chowei H, Saito T, Nakano N, Hayashi Y, Takeuchi M, Makita Z. Immunohistochemical distribution of the receptor for advanced glycation end products in neurons and astrocytes in Alzheimer's disease. *Brain Res.* 2001; 888:256–262.

Sasya M, Devi KSS, Babu JK, Balaguru Rayappan JB, Krishnan UM. Metabolic Syndrome-An Emerging Constellation of Risk Factors: Electrochemical Detection Strategies. *Sensors (Basel).* 2019; 20(1):103.

Satil F, Azcan N, Baser KHC. Fatty acid composition of pistachio nuts in Turkey. *Chem Nat Compd.* 2003; 39: 322–324.

Sauder KA, McCrea CE, Ulbrecht JS, Kris-Etherton PM, West SG. Effects of pistachios on the lipid/lipoprotein profile, glycemic control, inflammation, and endothelial function in type 2 diabetes: a randomized trial. *Metabolism.* 2015; 64(11): 1521–1529.

Sauder KA, McCrea CE, Ulbrecht JS, Kris-Etherton PM, West SG. Pistachio Nut Consumption Modifies Systemic Hemodynamics, Increases Heart Rate Variability, and Reduces Ambulatory

Blood Pressure in Well-Controlled Type 2 Diabetes: a Randomized Trial. *J Am Heart Assoc*. 2014; 3(4): e000873.

Sena CM, Pereira AM, Seiça R. Endothelial dysfunction - a major mediator of diabetic vascular disease. *Biochim Biophys Acta*. 2013;1832(12):2216-31.

Shabalala SC, Dludla PV, Mabasa L, Kappo AP, Basson AK, Pheiffer C, Johnson R. The effect of adiponectin in the pathogenesis of non-alcoholic fatty liver disease (NAFLD) and the potential role of polyphenols in the modulation of adiponectin signalling. *Biomed Pharmacother*. 2020; 131:110785.

Sheridan MJ, Cooper JN, Erario M, Cheifetz CE. Pistachio nut consumption and serum lipid levels. *J Am Coll Nutr.* 2007; 26:141-148.

Shivakumar N, Jackson AA, Courtney-Martin G, Elango R, Ghosh S, Hodgkinson S, Xipsiti M, Lee WTK, Kurpad AV, Tomé D. Protein Quality Assessment of Follow-up Formula for Young Children and Ready-to-Use Therapeutic Foods: Recommendations by the FAO Expert Working Group in 2017. *J Nutr.* 2020;150(2):195-201.

Siino V, Amato A, Di Salvo F, Caldara GF, Filogamo M, James P, Vasto S. Impact of diet-induced obesity on the mouse brain phosphoproteome. *J Nutr Biochem*. 2018; 58:102-109.

Silva Figueiredo P, Carla Inada A, Marcelino G, Maiara Lopes Cardozo C, de Cássia Freitas K, de Cássia Avellaneda Guimarães R, Pereira de Castro A, Aragão do Nascimento V, Aiko Hiane P. Fatty Acids Consumption: The Role Metabolic Aspects Involved in Obesity and Its Associated Disorders. *Nutrients.* 2017; 9(10): pii: E1158.

Simes DC, Viegas CSB, Araújo N, Marreiros C. Vitamin K as a Diet Supplement with Impact in Human Health: Current Evidence in Age-Related Diseases. *Nutrients.* 2020; 12(1): 138.

Singh S, Dharamveer Kulshreshtha M. Pharmacological approach of Pistacia Vera fruit to assess learning and memory potential in chemically-induced memory impairment in mice. *Cent Nerv Syst Agents Med Chem.* 2019; 19: 125-132.

Singh-Manoux A, Dugravot A, Shipley M, Brunner EJ, Elbaz A, Sabia S, Kivimakib M. Obesity trajectories and risk of dementia: 28 years of follow-up in the Whitehall II Study. *Alzheimers Dement.* 2018; 14(2): 178–186.

Siscovick D, Tucker KL, Ouyang P, Abbasi SA, Danielson K, Jerosch-Herold M, Mozaffarian D. Diet and adipose tissue distributions: The Multi-Ethnic Study of Atherosclerosis. *Nutr Metab Cardiovasc Dis*. 2016; 26:185-193.

Skurk T, Alberti-Huber C, Herder C, Hauner H. Relationship between adipocyte size and adipokine expression and secretion. *J Clin Endocrinol Metab.* 2007; 92: 1023-1033.

Smeriglio A, Denaro M, Barreca D, Calderaro A, Bisignano C, Ginestra G et al. In Vitro Evaluation of the Antioxidant, Cytoprotective, and Antimicrobial Properties of Essential Oil from *Pistacia vera* L. Variety Bronte Hull. *Int J Mol Sci*. 2017; 18(6): 1212.

Sokoła-Wysoczańska E, Wysoczański T, Wagner J, Czyż K, Bodkowski R, Lochyński S, Patkowska-Sokoła B. Polyunsaturated Fatty Acids and Their Potential Therapeutic Role in Cardiovascular System Disorders—A Review. *Nutrients*. 2018; 10(10): 1561.

Solfrizzi V, Frisardi V, Seripa D, Logroscino G, Imbimbo BP, D'Onofrio G, Addante F, Sancarlo D, Cascavilla L, Pilotto A, Panza F. Mediterranean diet in predementia and dementia syndromes. *Curr. Alzheimer. Res.* 2011; 8: 520-542.

Spyridopoulou K, Tiptiri-Kourpeti A, Lampri E, Fitsiou E, Vasileiadis S, Vamvakias M, Bardouki H, Goussia A, Malamou-Mitsi V, Panayiotidis MI, Galanis A, Pappa A, Chlichlia K. Dietary mastic oil extracted from pistacia lentiscus var. Chia suppresses tumor growth in experimental colon cancer models. *Sci Rep*. 2017; 7: 3782.

Steiner JL, Murphy EA, McClellan JL, Carmichael MD, Davis JM. Exercise training increases mitochondrial biogenesis in the brain. *J Appl Physiol.* 2011; 111: 1066-1071.

Stern JH, Rutkowski JM, Scherer PE. Adiponectin, Leptin, and Fatty Acids in the Maintenance of Metabolic Homeostasis through Adipose Tissue Crosstalk. *Cell Metab*. 2016; 23(5):770-84.

Storz MA. Is there a lack of support for whole-food, plant-based diets in the medical community? *Perm J*. 2018; 23: 18-068.

Stranahan AM, Norman ED, Lee K, Cutler RG, Telljohann RS, Egan JM, Mattson MP. Diet-induced insulin resistance impairs hippocampal synaptic plasticity and cognition in middle-aged rats. *Hippocampus.* 2008; 18: 1085-1088.

Suárez M, Boqué N, Del Bas JM, Mayneris-Perxachs J, Arola L, Caimari A. Mediterranean Diet and Multi-Ingredient-Based Interventions for the Management of Non-Alcoholic Fatty Liver Disease. *Nutrients.* 2017; 9: pii: E1052.

Swardfager W, Lanctot K, Rothenburg L, Wong A, Cappell J, Herrmann N. A meta-analysis of cytokines in Alzheimer's disease. *Biological psychiatry*. 2010; 68:930–941.

Tafti LD, Shariatpanahi SM, Damghani MM, Javadi B. Traditional persian topical medications for gastrointestinal diseases. *Iran J Basic Med Sci*. 2017; 20: 222-241.

Tan SY, Dhillon J, Mattes RD. A review of the effects of nuts on appetite, food intake, metabolism, and body weight. *Am J Clin Nutr*. 2014; 100: 412S–422S.

Taniguchi CM, Emanuelli B, Kahn CR. Critical nodes in signalling pathways: insights into insulin action. *Nat Rev Mol Cell Biol.* 2006; 7:85–96.

Tapiero H, Townsend DM, Tewb KD. The role of carotenoids in the prevention of human pathologies. *Biomed Pharmacother*. 2004; 58(2): 100–110.

Terwel D, Muyllaert D, Dewachter I, Borghgraef P, Croes S, Devijver H, Leuven FV. Amyloid Activates GSK-3β to Aggravate Neuronal Tauopathy in Bigenic Mice. *Am J Pathol.* 2008; 172(3): 786–798.

Terzo S, Baldassano S, Caldara GF, Ferrantelli V, Lo Dico G, Mulè F, Amato A. Health benefits of pistachios consumption. *Nat Prod Res*. 2019; 33:715-726.

Terzo S, Caldara GF, Ferrantelli V, Puleio R, Cassata G, Mulè F Amato A. Pistachio Consumption Prevents and Improves Lipid Dysmetabolism by Reducing the Lipid Metabolizing Gene Expression in Diet-Induced Obese Mice. *Nutrients* 2018a; 10:1857

Terzo S, Mulè F, Caldara GF, Baldassano S, Puleio R, Vitale M, Cassata G, Ferrantelli V, Amato A. Pistachio consumption alleviates inflammation and improves gut microbiota composition in mice fed a high-fat diet. *Int J Mol Sci.* 2020: 21: pii: E365.

Thevaranjan N, Puchta A, Schulz C, Naidoo A, Szamosi JC, Verschoor CP, Loukov D, Schenck LP, Jury J, Foley KP, Schertzer JD, Larché MJ, Davidson DJ, Verdú EF, Surette MG, Bowdish DE.

Age-Associated Microbial Dysbiosis Promotes Intestinal Permeability, Systemic Inflammation, and Macrophage Dysfunction. *Cell Host Microbe* 2017; 21: 455-466.

Tian Y, Su L, Wang J, Duan X, Jiang X. Fruit and vegetable consumption and risk of the metabolic syndrome: A meta-analysis. *Public Health Nutr*. 2018; 21: 756-765.

Tokuşoglu O, Unal MK, Yemiş F. Determination of the phytoalexin resveratrol (3,5,4'-trihydroxystilbene) in peanuts and pistachios by high-performance liquid chromatographic diode array (HPLC-DAD) and gas chromatography-mass spectrometry (GC-MS). *J Agric Food Chem*. 2005; 53:5003-5009.

Tomaino A, Martorana M, Arcoraci T, et al. Antioxidant activity and phenolic profile of pistachio (*Pistacia vera L.*, variety Bronte) seeds and skins. *Biochimie*. 2010; 92: 1115–1122.

Trayhurn P, Wood IS. Adipokines: inflammation and the pleiotropic role of white adipose tissue. *Br J Nutr* 2004; 92: 347-355.

Tsigos C, Kyrou I, Chala E, Tsapogas P, Stavridis JC, Raptis SA, Katsilambros N. Circulating tumor necrosis factor alpha concentrations are higher in abdominal versus peripheral obesity. *Metabolism.* 1999; 48(10):1332-5.

Tucsek Z, Toth P, Sosnowska D, Gautam T, Mitschelen M, Koller A, Szalai G, Sonntag WE, Ungvari Z, Csiszar A. Obesity in aging exacerbates blood-brain barrier disruption, neuroinflammation, and oxidative stress in the mouse hippocampus: effects on expression of genes involved in beta-amyloid generation and Alzheimer's disease. *J Gerontol A Biol Sci Med Sci*. 2014; 69:1212–1226.

Ukhanova M, Wang X, Baer DJ, Novotny JA, Fredborg M, Mai V. Effects of almond and pistachio consumption on gut microbiota composition in a randomised cross-over human feeding study. *Br J Nutr*. 2014; 111: 2146-2152.

Uttara B, Singh AV, Zamboni P, Mahajan RT. Oxidative stress and neurodegenerative diseases: a review of upstream and downstream antioxidant therapeutic options. *Curr. Neuropharmacol*. 2009; 7: 65-74.

Vadivel V, Kunyanga CN, Biesalski HK. Health benefits of nut consumption with special reference to body weight control. *Nutrition.* 2012; 28:1089-1097.

Vague J. Sex differentiation, a determining factor in the forms of obesity. *Presse Med*. 1947; 55:339-340.

Vaidya HB, Gangadaran S, Cheema SK. A high fat-high sucrose diet enriched in blue mussels protects against systemic inflammation, metabolic dysregulation and weight gain in C57BL/6 mice. *Food Res Int*. 2017; 100:78-85.

Vasto S, Barera A, Rizzo C, Di Carlo M, Caruso C, Panotopoulos G. Mediterranean Diet and Longevity: An Example of Nutraceuticals? *Curr Vasc Pharmacol* 2014a; 12: 735-788.

Vasto S, Buscemi S, Barera A, Di Carlo M, Accardi G, Caruso C. Mediterranean diet and healthy ageing: A sicilian perspective. *Gerontology*. 2014b; 60: 508-518.

Vázquez Cisneros LC, Martínez Moreno AG, López-Espinoza A, Espinoza-Gallardo AC. Effect of the fatty acid composition of meals on postprandial energy expenditure: a systematic review. *Rev Assoc Med Bras.* 2019; 65(7):1022-1031.

Verdile G, Keane KN, Cruzat VF, Medic S, Sabale M, Rowles J, Wijesekara N, Martins RN, Fraser PE, Newsholme. Inflammation and Oxidative Stress: The Molecular Connectivity between Insulin Resistance, Obesity, and Alzheimer's Disease. *Mediators Inflamm.* 2015; 2015: 105828.

Vilahur G, Ben-Aicha S, Diaz E, Badimon L, Padro T. Phytosterols and inflammation. *Curr Med Chem.* 2019; 26:6724-6734.

Wainwright P and Byrne CD. Bidirectional Relationships and Disconnects between NAFLD and Features of the Metabolic Syndrome. *Int J Mol Sci*. 2016; 17(3): 367.

Wang S, Fortier M, Greenberg AS, Obin MS. Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. *J Lipid Res.* 2005; 46: 2347-2355.

Wang X, Li Z, Liu Y, Lv X, Yang W. Effects of pistachios on body weight in Chinese subjects with metabolic syndrome. *Nutr J*. 2012; 11: 20.

Wang Y, Fei Y, Liu L, Xiao Y, Pang Y, Kang J, Wang Z. Polygonatum odoratum Polysaccharides Modulate Gut Microbiota and Mitigate Experimentally Induced Obesity in Rats. *Int J Mol Sci.* 2018; 19:3587.

West AP, Khoury-Hanold W, Staron M, Tal MC, Pineda CM, Lang SM, Bestwick M, Duguay BA, Raimundo N, MacDuff DA, Kaech SM, Smiley JR, Means RE, Iwasaki A, Shadel GS. Mitochondrial DNA stress primes the antiviral innate immune response. *Nature* 2015; 520: 553-557.

West SG, Gebauer SK, Kay CD, Bagshaw DM, Savastano DM, Diefenbach C, Kris-Etherton PM. Diets containing pistachios reduce systolic blood pressure and peripheral vascular responses to stress in adults with dyslipidemia. *Hypertension.* 2012; 60(1):58-63.

Wilson DW, Nash P, Buttar HS, Griffiths K, Singh R, De Meester F, Horiuchi R, Takahashi T. The Role of Food Antioxidants, Benefits of Functional Foods, and Influence of Feeding Habits on the Health of the Older Person: An Overview. *Antioxidants (Basel)* 2017; 6(4): 81.

Wisse BE. The inflammatory syndrome: the role of adipose tissue cytokines in metabolic disorders linked to obesity. *J Am Soc Nephrol.* 2004; 15(11):2792-800.

Wu H, Ballantyne CM. Metabolic Inflammation and Insulin Resistance in Obesity. *Circ Res.* 2020; 126: 1549-1564.

Wu N, Siow YL. Induction of hepatic cyclooxygenase-2 by hyperhomocysteinemia via nuclear factor-kappaB activation. *Am J Physiol Regul Integr Comp Physiol*. 2009; 297:1086-1094.

Wu S, Hu R, Nakano H, Chen K, Liu M, He X, Zhang H, He J, Hou DX. Modulation of Gut Microbiota by Lonicera caerulea L. Berry Polyphenols in a Mouse Model of Fatty Liver Induced by High Fat Diet. *Molecules* 2018; 23: pii: E3213.

Xia SF, Le GW, Wang P, Qiu YY, Jiang YY, Tang X. Regressive Effect of Myricetin on Hepatic Steatosis in Mice Fed a High-Fat Diet. *Nutrients.* 2016; 8: pii: E799.

Xu J, Mahowald MA, Ley RE, Lozupone CA, Hamady M, Martens EC, Henrissat B, Coutinho PM, Minx P, Latreille P, Cordum H, Van Brunt A, Kim K, Fulton RS, Fulton LA, Clifton SW, Wilson

RK, Knight RD, Gordon JI. Evolution of symbiotic bacteria in the distal human intestine. *PLoS Biol*. 2007; 5: e156.

Yang C, Deng Q, Xu J, Wang X, Hu C, Tang H, Huang F. Sinapic acid and resveratrol alleviate oxidative stress with modulation of gut microbiota in high-fat diet-fed rats. *Food Res Int*. 2019; 116:1202-1211.

Yayeh T, Hong M, Jia Q, Lee YC, Kim HJ, Hyun E, Kim TW, Rhee MH. Pistacia chinensis inhibits no production and upregulates ho-1 induction via pi-3k/akt pathway in lps stimulated macrophage cells. *Am J Chin Med*. 2012; 40: 1085–1097.

Yuan H, Ma Q, Ye L, Piao G. The Traditional Medicine and Modern Medicine from Natural Products. *Molecules*. 2016; 21(5): 559.

Zhang B, Lu XL, Song YH, Shi HT, Li J, Geng Y. Changes in the intestinal microenvironment during development of alcoholic fatty liver disease and related effects of probiotic therapy. *Zhonghua Gan Zang Bing Za Zhi* 2012; 20: 848-852.

Zhang J, Kris-Etherton PM, Thompson JT, Vanden Heuvel JP. Effect of pistachio oil on gene expression of IFN-induced protein with tetratricopeptide repeats 2: A biomarker of inflammatory response. *Mol Nutr Food Res*. 2010; 54: S83-92.

Zheng D, Liwinski T, Elinav E. Inflammasome activation and regulation: toward a better understanding of complex mechanisms. *Cell Discov*. 2020; 6:36.

Zhou D, Chen YW, Zhao ZH, Yang RX, Xin FZ, Liu XL, Pan Q, Zhou H, Fan JG. Sodium butyrate reduces high-fat diet-induced non-alcoholic steatohepatitis through upregulation of hepatic GLP-1R expression. *Exp Mol Med*. 2018; 50: 157.

Zhu Q, Gao R, Wu W, Qin H. The role of gut microbiota in the pathogenesis of colorectal cancer. *Tumour Biol.* 2013; 34: 1285-1300.

Zhu Y, Huang X, Zhang Y, Wang Y, Liu Y, Sun R, Xia M. Anthocyanin supplementation improves HDL-associated paraoxonase 1 activity and enhances cholesterol efflux capacity in subjects with hypercholesterolemia. *J Clin Endocrinol Metab*. 2014; 99(2):561-9.

Zhuang ZJ, Shan CW, Li B, Pang MX, Wang H, Luo Y, Liu YL, Song Y, Wang NN, Chen SH, Shi JP, Lv GY. Linarin Enriched Extract Attenuates Liver Injury and Inflammation Induced by High-Fat High-Cholesterol Diet in Rats. *Evid Based Complement Alternat Med*. 2017; 2017: 4701570.

Rochlani Y, Pothineni NV, Kovelamudi S, Mehta JL. Metabolic syndrome: pathophysiology, management, and modulation by natural compounds. *Ther Adv Cardiovasc Dis*. 2017; 11:215-225.